Regulation of biliary cholesterol secretion and reverse cholesterol transport

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Contents

Chapter 1

Introduction

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Abstract

Biliary cholesterol secretion is a process important for two major disease complexes, atherosclerotic cardiovascular disease and cholesterol gallstone disease. With respect to cardiovascular disease, biliary cholesterol secretion is regarded the final step for the irreversible elimination of cholesterol originating from cholesterol-laden macrophage foam cells in the vessel wall in a pathway coined reverse cholesterol transport. On the other hand, cholesterol hypersecretion into the bile is considered the main pathophysiological determinant of cholesterol gallstone formation. This review summarizes current knowledge on the origins of cholesterol secreted into the bile as well as the relevant processes and transporters involved. Next to the established ATP-binding cassette (ABC) transporters mediating the biliary secretion of bile acids (ABCB11), phospholipids (ABCB4) and cholesterol (ABCG5/G8), special attention is given to emerging proteins that modulate or mediate biliary cholesterol secretion. In this regard, the potential impact of the phosphatidylserine flippase ATP8B1, the Niemann Pick C1-like protein 1 (NPC1L1) that mediates cholesterol absorption and the high density lipoprotein (HDL) cholesterol uptake receptor scavenger receptor BI (SR-BI) is discussed.

What is the importance of biliary cholesterol secretion?

The liver plays a central role in cholesterol metabolism (Figure 1). Hepatocytes not only express a number of different lipoprotein receptors including the low density lipoprotein receptor (LDLR), the LDLR-related protein (LRP) and the scavenger receptor class B type I (SR-BI) that enable them to take up cholesterol from virtually all lipoprotein subclasses, cholesterol is also synthesized de novo within the liver in a regulated fashion [1]. In addition to these input pathways into the hepatic cholesterol pool, the liver is equipped to actively secrete cholesterol via two different routes: (1) within triglyceride-rich very low density lipoproteins (VLDL) [2, 3], thereby supplying peripheral cells with fatty acids, fat soluble vitamins and cholesterol; (2) by secretion into the bile either directly as free cholesterol or after conversion into bile acids, thereby providing a means of irreversible elimination of cholesterol from the body via the feces [4, 5]. The different hepatic cholesterol fluxes are interrelated, however, some are also kept remarkably separate, as will be discussed later in this review.
Biliary cholesterol secretion itself is directly linked to two major disease complexes with a high relevance for health care systems worldwide, atherosclerotic cardiovascular disease (CVD) and gallstone disease. In atherosclerotic CVD, biliary cholesterol secretion is considered the final step in the completion of the reverse cholesterol transport (RCT) pathway [6, 7]. The term RCT comprises the transport of peripheral cholesterol back to the liver for excretion into bile, most importantly cholesterol accumulating within macrophage foam cells in atherosclerotic lesions [8]. For RCT an enhanced biliary secretion of cholesterol originating from peripheral pools relevant for CVD is desirable. On the other hand, increased biliary cholesterol secretion is related to biliary cholesterol supersaturation, which is an important determinant for the formation of cholesterol gallstones that constitute more than 90% of all gallstones [9, 10]. Notably, both, CVD [11, 12] and gallstone disease [13], also have a strong inflammatory component that plays an important role in the pathogenesis of these diseases. However, an in-depth understanding of the metabolic processes and transporters involved in the regulation of biliary cholesterol secretion is important and might conceivably reveal relevant targets for the treatment of CVD as well as cholesterol gallstone disease.

What is the origin of cholesterol secreted into bile?

The most relevant source of cholesterol secreted into the bile is cholesterol derived from plasma lipoproteins, a less relevant source represents cholesterol originating from de novo synthesis or hydrolysis of stored cholesteryl ester [14]. HDL appears to be the preferential contributor for cholesterol secreted into bile [15]. In humans with a bile fistula, cholesterol originating from HDL was more rapidly appearing in bile compared with LDL cholesterol [16]. Additional evidence for a more prominent role of HDL over LDL came from experiments demonstrating that biliary cholesterol

secretion remained essentially unchanged when specifically plasma LDL cholesterol levels were reduced by 26% by means of LDL apheresis [17]. On the contrary, reduction in plasma LDL cholesterol resulted in a consecutive decrease in biliary bile acid secretion [17], thereby lending further experimental evidence to older literature suggesting a metabolic compartmentalization of hepatic cholesterol pools with regard to bile acid synthesis versus direct biliary secretion [18, 19]. However, definitive studies exploring the underlying metabolic pathways are still lacking.
It is, however, important to note, that modulation of plasma HDL cholesterol levels does not influence mass biliary cholesterol secretion, since ABCA1 knockout mice [20] as well as apoA-I knockout mice [21, 22] display unaltered biliary cholesterol secretion rates. These data indicate that specific intrahepatic metabolic and transport processes are most relevant and rate-limiting for biliary secretion of cholesterol.

How does cholesterol originating from lipoproteins or synthesized intracellularly get to the canalicular membrane?

The hepatocyte is a polarized cell with a basolateral (sinusoidal) and an apical (canalicular) plasma membrane. Uptake of cholesterol from the plasma compartment occurs on the basolateral site (Figure 1), while biliary cholesterol secretion is an apical process (Figure 2). Hepatic cholesterol synthesis is carried out intracellularly. This polarization implies that means of transport must exist for exogenous as well as endogenously synthesized cholesterol to physically reach the site where the biliary secretion process takes place.
Of note, cholesterol is also used for the synthesis of bile acids, and a number of factors modulating bile acid synthesis impact on hepatic cholesterol homeostasis. However, the regulation of bile acid synthesis will not be discussed herein, we refer to recent comprehensive reviews covering this topic [23, 24].
The detailed mechanisms of intracellular cholesterol trafficking in hepatocytes are not understood thus far, especially also not in their relation to biliary cholesterol secretion. However, for a couple of known transport proteins studies have been performed to test the effect of gain- or loss-of-function manipulations on the biliary cholesterol secretion phenotype. A prominent example of such a protein is the Niemann-Pick type C protein 1 (NPC1) that plays a role in the intracellular trafficking of lipoprotein-derived cholesterol [25, 26]. Mice lacking NPC1 expression had 37% higher biliary cholesterol secretion rates on chow diet compared with controls [27]. However, when fed a 2% cholesterol containing diet, biliary cholesterol secretion was significantly decreased in NPC1 knockout mice, while it increased 3.7-fold in wild-type controls [27]. In turn, hepatic overexpression of NPC1 increased biliary cholesterol secretion by approximately 2-fold in chow-fed wild-type and high cholesterol diet-fed NPC1 knockout mice, while no effect was seen in wild-type mice fed a 2% cholesterol diet [27]. Although the interpretation of this latter result is not straightforward, these data indicate that NPC1 might be important in regulating the

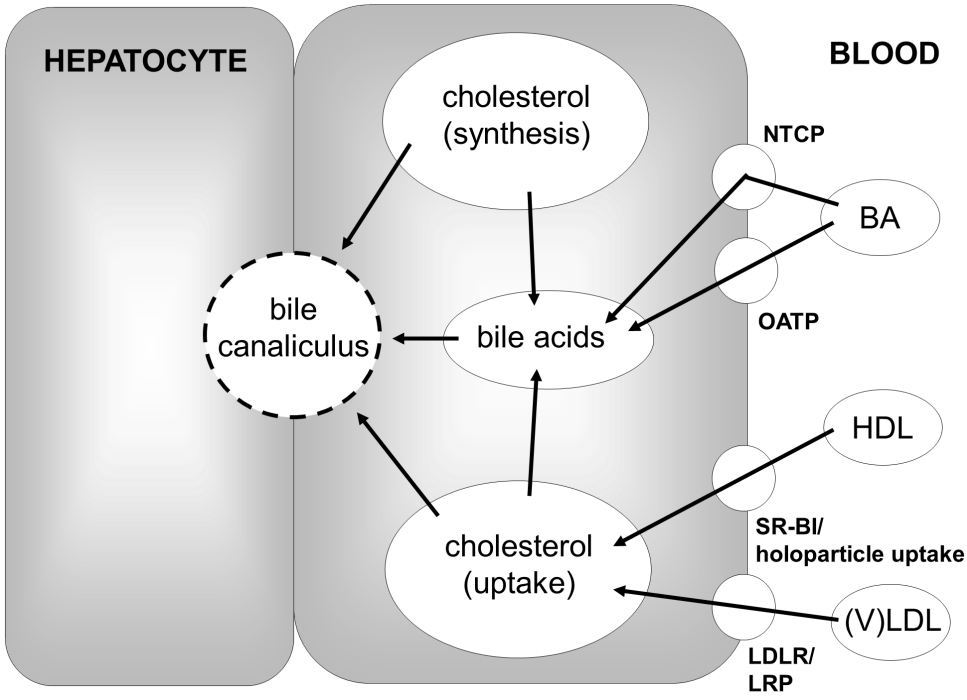

Figure 1: Overview of hepatic cholesterol metabolism in relation to biliary sterol secretion. BA, bile acid; HDL, high-density lipoprotein; LDLR, low-density lipoprotein receptor; LRP, LDLR-related protein; NTCP, Na+ taurocholate co-transporting peptide; OATP, organic anion transport polypeptides; SR-BI, scavenger receptor class B type I; (V)LDL, (very) low-density lipoprotein.

availablility of cholesterol at the canalicular membrane for the biliary secretion process. Also NPC2, that plays a very similar role in intracellular cholesterol trafficking [28], is expressed in liver, is detectable in bile, and might be involved in the transport of cholesterol destined for biliary secretion. However, thus far no mechanistic studies have been performed, only a significant increase of hepatic NPC2 expression in gallstone-susceptible versus gallstone-resistant mouse strains has been reported [29]. In addition, also the expression of another carrier protein the sterol carrier protein-2 (SCP2) impacts biliary cholesterol secretion, with SCP2 overexpression increasing biliary secretion of cholesterol [30, 31] and decreased SCP2 expression having the opposite effect [32]. Members of the steroidogenic acute regulatory (StAR) protein family of cholesterol transport molecules might also represent candidates to influence biliary cholesterol secretion [33, 34]. However, thus far it has only been shown that StARD1 overexpression increases bile acid synthesis [35], while the absence of StARD3 in knockout mice had no impact on biliary sterol secretion [36]. In addition to intracellular cholesterol transport proteins, also enzymes modulating the amount of free cholesterol present within a hepatocyte could be expected to impact biliary cholesterol secretion. While this was shown to be the case for the neutral cholesteryl

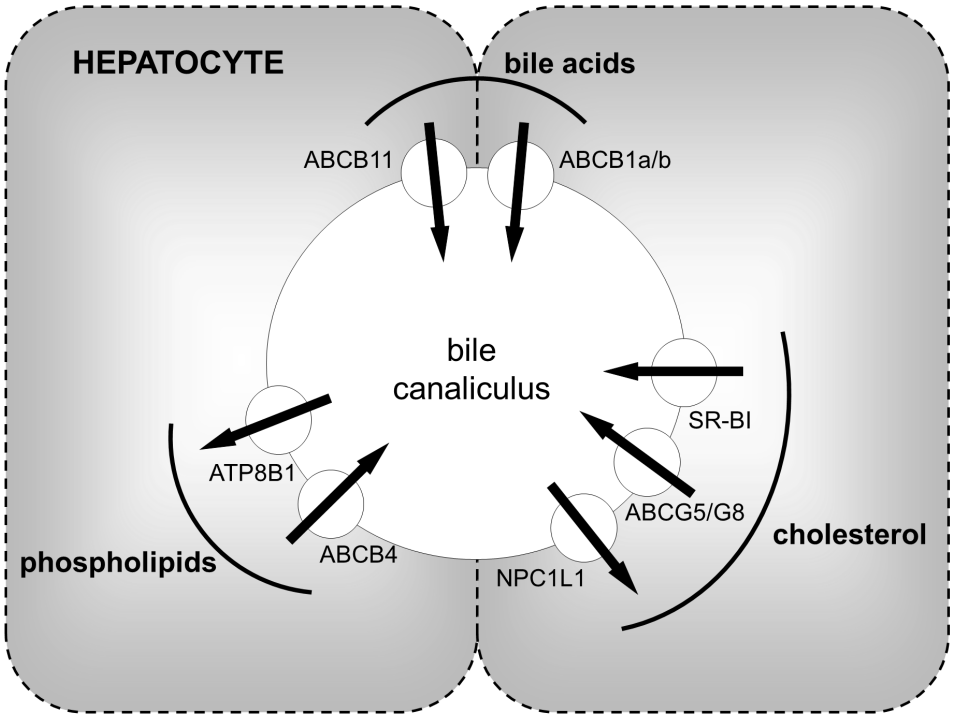

Figure 2: Overview of canalicular transporters/proteins that are involved in biliary bile acid, cholesterol and phospolipid secretion. ABC, ATP-binding cassette transporter; ATP8B1, ATPase class I type 8B member 1; NPC1L1, Niemann-Pick C1-like protein 1; SR-BI, scavenger receptor class B type I.

ester hydrolase [37], decreasing ACAT2 expression had no effect on biliary cholesterol output [38].

Which transporters are involved in biliary cholesterol secretion?

On the apical membrane several transporters are directly involved in the biliary cholesterol secretion process. Transporter expression and activity is regulated by transcriptional as well as posttranscriptional mechanisms. Important transcriptional regulators are the nuclear hormone receptors liver X receptor (LXR; two isoforms LXRalpha, NR1H3, and LXRbeta, NR1H2) and farnesoid X receptor (FXR, NR1H4), among others. For the specifics of LXR- and FXR-mediated gene regulation we refer to recent articles dealing with these topics [23, 39-41]. In general, LXR is a nuclear receptor activated by oxysterols and functions as a sterol sensor exerting control on cholesterol metabolism [39, 40]. As a general scheme, LXR activation stimulates metabolic processes favoring cholesterol elimination from the body, e.g. by increasing biliary cholesterol secretion via increased expression of ABCG5/G8 [42] (see be-

low) or by increasing the conversion of cholesterol to bile acids via increased expression of Cyp7A1, at least in rodents [43].
On the other hand, FXR is the nuclear receptor activated by bile acids and orchestrates an adaptive response of the hepatocyte to altered bile acid levels [23, 41]. E.g. in cholestatic conditions, FXR induces a downregulation of bile acid synthesis [23], a reduction in bile acid uptake into hepatocytes by decreasing the expression of the cellular uptake receptors [23] and an increased biliary secretion of bile acids by increasing the expression of specific transporter such as ABCB11 [44] (see below).
In general, biliary bile acid secretion is the main driving force for the secretion of phospholipids and cholesterol [45]. Infusion of bile acids results in a dose-dependent increase in biliary cholesterol secretion [46]. However, cholesterol secretion into the bile also critically depends on functional biliary phospholipid secretion which is required for the formation of mixed micelles [46]. This is evidenced by the Abcb4 knockout mouse, that in the absence of this key biliary phospholipid transporter almost completely lacks biliary cholesterol secretion [47, 48]. The following transporters are specifically involved in these processes:

ABCB4
ABCB4 has historically been named multi-drug resistance P-glycoprotein 2 (Mdr2). ABCB4 functions as a phosphatidylcholine (PC) floppase translocating PC from the inner to the outer leaflet of the canalicular membrane [45]. Biliary cholesterol secretion is fully dependent on the functionality of ABCB4 and thereby biliary phospholipid secretion. In the absence of Abcb4, biliary cholesterol secretion is virtually absent [47, 48]. Furthermore, addition of PC decreases the toxic effects of bile acids on the canalicular membrane, and Abcb4 knockout mice as well as patients lacking this transporter develop a progressive liver disease [49, 50].

ATP8B1
ATP8B1 is a P-type ATPase that flips phosphatidylserine (PS) from the outer to the inner leaflet of the canalicular membrane resulting in a reduction in the PS content and a consecutive increase in the sphingomyelin (SM) content of the outer canalicular leaflet [51, 52]. In humans, mutations in ATP8B1 cause severe chronic or periodic cholestatic liver disease [53, 54]. A mutation resulting in severe liver disease is the glycine to valine substitution at amino acid 308 (G308V) [54]. In Atp8b1G308V/G308V knock-in mice Atp8b1 is almost completely absent, which causes enhanced biliary excretion of PS and also cholesterol, mainly due to decreased rigidity of the outer canalicular leaflet (see below) [52, 55].

ABCB11
ABCB11 is classically referred to as the bile salt export pump (BSEP; also known as sister of P-glycoprotein, spgp) and is mediating biliary secretion of bile acids [44, 56]. A 2-fold increase in ABCB11 expression in transgenic mice resulted in increased biliary output of bile acids [57]. Notably expression of other biliary transporters re-

mained unaltered providing additional evidence that biliary bile acid secretion is the driving force for the secretion of the other lipid species into bile [57]. However, in Abcb11 knockout mice, there is still substantial residual biliary bile acid secretion and the expression of Mdr1a (Abcb1a) and Mdr1b (Abcb1b) was found to be up-regulated indicating a potential compensation mechanism [58]. Subsequently, triple knockout mice lacking all three transporters have been generated to prove this concept, and indeed these mice develop an extreme cholestatic phenotype [59].

How do these transporters work together in the process of bile formation? First, the specific properties of the canalicular membrane need to be considered, since this has to fulfill two key functions: (i) withstand extremely high detergent (=bile acid) concentrations that are able to basically solubilize normal plasma membranes resulting in cell death, and (ii) still allow for regulated secretion of the different bile components. The answer to these questions seems to lie in the asymmetry of the canalicular membrane with a high content of sphingomyelin and cholesterol in the outer leaflet [60]. In membranes composed of glycerophospholipids (PC, PS and phosphatidylethanolamine) lipids are loosely packed, the so-called "liquid disordered" phase [61, 62]. Addition of sphingomyelin and cholesterol induces a more rigid membrane structure, the so-called "liquid ordered" phase that is not allowing for the intercalation of detergents and thereby the membrane is rendered increasingly resistant against detergents [61, 63, 64]. The task of ATP8B1 in this model would be to preserve the rigid structure of the outer canalicular leaflet by inward flipping of PS. The amphipatic bile acids are actively excreted into the canalicular lumen by ABCB11, where they form simple micelles in the aqueous environment of the bile [9, 46]. Phospholipids are translocated to the outer leaflet of the canalicular membrane by ABCB4 where they are added to the simple micelles resulting in mixed micelles [9, 46]. However, the precise mechanism how this occurs is still elusive. Subsequently, cholesterol is then taken up into these mixed micelles [9, 46]. The authentic cholesterol transporters that play a role in this process are discussed in the following.

ABCG5/G8

The transport system contributing quantitatively the major amount of cholesterol secretion into the bile is the obligate heterodimer transporter pair ABCG5/ABCG8 [65-67]. These are expressed almost exclusively in the liver and the intestine and respective mutations have been identified as the disease substrate for sitosterolemia, a rare autosomal recessive disorder which is characterized by the accumulation of plant sterols in blood and tissues [68, 69]. This accumulation is caused by increased sterol absorption from the diet and decreased biliary sterol secretion [70, 71]. In the intestine, ABCG5/G8 secrete absorbed plant sterols back into the intestinal lumen, while on the bile canaliculus ABCG5/G8 mediate biliary plant sterol as well as cholesterol secretion into bile [70, 71]. Since in healthy individuals, plasma plant sterol levels are very low, ABCG5/G8 represent under physiological conditions mainly a transport system for cholesterol. This is mirrored by the fact that biliary cholesterol

secretion in Abcg5 and/or Abcg8 knockout mice is reduced by 75% [71-73]. In turn, transgenic overexpression of ABCG5/G8 resulted in an increase in biliary cholesterol secretion that was over a wide range directly proportional to the copy numbers of the transgene, indicating that under these conditions neither delivery of cholesterol to the transporters nor the level of cholesterol acceptors within the bile are rate-limiting [74]. In addition, ABCG5 and ABCG8 are targets of the nuclear hormone receptor LXRα and the increase in biliary cholesterol secretion observed upon LXR activation with endogenus or synthetic ligands depends largely on functional ABCG5/G8 expression [75]. Interestingly, also the increasing effects of cholate as well as diosgenin on biliary cholesterol secretion depend on the functional expression of Abcg5/g8 and subsequently are not seen in mice lacking either or both of the transporters [74, 76]. Notably, there are as yet ill-defined ABCG5/G8-independent pathways of biliary cholesterol secretion [77] (see also below).

With relevance to atherosclerotic CVD, when human ABCG5/G8 transgenic mice overexpressing the transgene in the liver as well as in the intestine were crossed into the atherosclerotic Ldlr-/- genetic background, these mice developed significantly less atherosclerosis compared with wild-type controls [78]. On the other hand, only hepatic overexpression of ABCG5/G8 does not alter atherosclerosis in Ldlr-/- as well as apoE-/- mice [79], unless intestinal cholesterol absorption is also reduced by ezetimibe [80]. With relevance to gallstone disease, recently specific mutations in ABCG5/G8, namely ABCG5 R50C and ABCG8 D19H, have been identified in humans and shown to increase the risk for cholesterol gallstone disease [81-83]. Since the role of ABCG5/G8 is to increase biliary cholesterol secretion and cholesterol gallstone formation requires increased amounts of cholesterol within bile, these respective mutations are likely to constitute a gain-of-function phenotype. However, since these association data were obtained in human studies, this concept still requires verification in an experimental setting that allows to decipher cause-effect relationships.

Although several studies have shown that the ABCG5/G8 heterodimer is a key component of biliary cholesterol secretion [70, 71], the molecular mechanism by which this transporter pair mediates biliary cholesterol secretion at the canalicular membrane has not been elucidated, yet. Mainly, this lack of insight is due to the unavailability of easy to study model systems such as polarized hepatocyte cell lines or simple and reliable methods to isolate and characterize pure apical and basolateral membranes. However, the currently most accepted model suggests that ABCG5/ABCG8 act as a liftase elevating cholesterol just sufficiently outside of the outer leaflet of the canalicular membrane to be extracted by mixed micelles [84].

NPC1L1

The Niemann-Pick C1-like protein 1 (NPC1L1) was originally identified as a key regulator of intestinal cholesterol absorption and the molecular target of the cholesterol absorption inhibitor ezetimibe [85, 86]. It is also highly expressed in human liver, however, not in rodents [87]. In human hepatocytes, NPC1L1 is localized to the canalicular membrane [88]. Similar to its role in the intestine, hepatic NPC1L1 ap-

parently facilitates the uptake of newly secreted biliary cholesterol [89]. In NPC1L1 transgenic mice with hepatic overexpression of the transgene a more than 90% decrease in biliary cholesterol concentration is observed without any effect on biliary phospholipid and bile acid levels. Interestingly, treatment with ezetimibe normalizes biliary cholesterol concentrations in hNPC1L1 transgenic animals [89]. Therefore, especially in humans ezetimibe supposedly reduces plasma cholesterol levels by inhibiting both intestinal as well as hepatic NPC1L1 activity. However, the regulation of this important cholesterol transporter and modifier of hepatic cholesterol secretion is still incompletely understood.

SR-BI

SR-BI has been characterized as receptor for HDL cholesterol, mediating bi-directional cholesterol flux, either efflux or selective uptake, depending on concentration gradients [90, 91]. In contrast to ABC transporters, SR-BI apparently requires no energy consumption [91]. SR-BI is mainly expressed in the liver and in steroidogenic tissue [90, 91]. In hepatocytes, SR-BI mediates the internalization of HDL cholesterol without the concomittant catabolism of HDL apolipoproteins [90, 91]. SR-BI is detectable in hepatocytes in vivo at both, the basolateral as well as the apical membrane [92, 93]. Since HDL-derived cholesterol is a major source of sterols designated for biliary secretion [15], these properties of SR-BI suggested a potential involvement in biliary cholesterol secretion.

Cholesterol uptake from HDL via SR-BI can be increased in two different ways, by modulating the properties of the ligand, namely HDL, as well as by changing the expression level of SR-BI. HDL modification by phospholipases decreases its phospholipid content, destabilizes the particle and makes the HDL cholesterol ester more susceptible towards SR-BI-mediated selective uptake [94-97]. Overexpression of two phospholipases relevant for human physiology in mice, namely group IIA secretory phospholipase A2 (sPLA2) [98, 99] and endothelial lipase (EL) [95, 97, 100], each resulted in a significant decrease in plasma HDL cholesterol levels and increased hepatic cholesterol levels without affecting SR-BI expression or membrane localization [94, 101]. Consecutive in vitro studies showed that SR-BI-mediated selective uptake from sPLA2- or EL-modified HDL was increased by 77% [94] and 129% [97], respectively. However, when biliary cholesterol secretion was measured in sPLA2 transgenic mice or in mice with adenovirus-mediated EL overexpression, these were essentially unchanged compared with controls [94, 101]. In addition, also the absence of hepatic lipase does not affect biliary cholesterol secretion, although in this study actual SR-BI-mediated uptake of HDL cholesterol was not quantitated [102]. Overall, these data demonstrate that biliary cholesterol secretion remains unaltered when only the ligand, HDL, but not SR-BI expression itself is modulated.

In contrast, changes in the hepatic expression level of SR-BI directly translate into altered biliary cholesterol secretion rates. SR-BI knockout mice have reduced biliary cholesterol secretion rates by 55% [103]. Consistent with these data, adenovirus-mediated SR-BI overexpression in the liver of wild-type mice increases gallbladder cho-

lesterol content [92] as well as biliary cholesterol secretion rates [93]. Interestingly, with relevance to disease hepatic SR-BI overexpression increases reverse cholesterol transport [104] and is protective against atherosclerotic CVD, even though plasma HDL cholesterol levels are decreased [105, 106]. In gallstone-susceptible mice, SR-BI hepatic expression was higher and associated with biliary cholesterol hypersecretion [107]. In addition, hepatic mRNA as well as protein expression of SR-BI was higher in Chinese patients with cholesterol gallstone disease compared with controls and even correlated with an increased cholesterol saturation index [108] suggesting that the link between SR-BI and biliary cholesterol secretion might be relevant for human pathophysiology as well. In this study, also increased expression of LXRα was noted in the livers of gallstone patients[108] and previous experimental work suggested that modulation of SR-BI expression results in altered expression of LXR target genes [109].

However, increased biliary cholesterol secretion in response to SR-BI overexpression was completely independent of LXR as evidenced by the use of knockout mice[93]. Surprisingly, this biological effect was also independent of functional expression of ABCG5/G8, since in Abcg5 knockout mice hepatic overexpression of SR-BI increased biliary cholesterol secretion rates to the levels of wild-type control mice and thereby normalized the cholesterol secretion deficit caused by absent Abcg5 expression [93]. To a certain extent canalicular SR-BI also acts independent of ABCB4, since hepatic overexpression of SR-BI in Abcb4 knockout mice resulted in significantly higher biliary cholesterol secretion, however, in terms of mass secretion this effect was minor, indicating that cholesterol secreted via SR-BI still requires mixed micelles as acceptors [93]. Interestingly, SR-BI overexpression augmented particularly canalicular expression of the receptor [93]. Since in SR-BI overexpression conditions increased canalicular SR-BI localization was associated with an increased cholesterol content of the canalicular membrane and higher biliary cholesterol secretion rates [93], conceivably this biological effect was mediated by SR-BI. SR-BI requires a cholesterol gradient for transport [91], which is, however, constantly provided within the canaliculus by the hepatopetal bile flow and the associated transport of cholesterol away from the hepatocyte. It would be interesting and physiologically relevant to explore whether SR-BI also contributes a significant part to the ABCG5/G8-independent biliary cholesterol secretion under steady-state conditions and not only has an effect in response to overexpression.

In addition, it is presently unclear how cholesterol secreted by SR-BI into the bile reaches the canalicular membrane. One possibility could be that SR-BI itself mediates this transport, since a transcytotic route of trafficking from the basolateral to the apical membrane has been proposed for SR-BI [110]. However, this pathway is dependent on microtubular function, and recent studies have demonstrated that SR-BI overexpression significantly increases biliary cholesterol secretion even under conditions when microtubuli function and transcytotic transport were efficiently abolished in hepatocytes in vivo [93]. Therefore, other, as yet not characterized pathways, are likely to contribute.

What directions should future research take?

In our view, there are a number of interesting and relevant questions that are unresolved and should be addressed by future research in the field. The contribution of SR-BI to the ABCG5/G8-independent biliary cholesterol secretion under steady-state conditions would be important to know. Relating to this but also extending beyond SR-BI, more work appears to be required to identify and delineate the intracellular pathways for cholesterol transport that contribute to biliary cholesterol secretion. In addition to the selective uptake pathway for HDL cholesterol that is mediated by SR-BI, also a holoparticle uptake pathway has been characterized that is responsible for approximately one fourth of the total HDL cholesterol taken up into the liver [97, 111-114]. However, if this pathway contributes cholesterol for biliary secretion is currently unknown. Expanding this question, there also appears to be room to assess the differential contribution of apoB-containing lipoproteins versus HDL to bile acid synthesis and the respective biliary secretion of cholesterol and bile acids. Further research on these pathways would not only be relevant to increase our mechanistic insights into pathophysiology but also to define and characterize potential novel therapeutic targets for the treatment of atherosclerotic CVD and cholesterol gallstone disease.

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Chapter 2

Scavenger receptor BI and ABCG5/G8 differentially impact biliary sterol secretion and reverse cholesterol transport in mice

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Abstract

Biliary lipid secretion plays an important role in gallstone disease and reverse cholesterol transport (RCT). Using Sr-bI/Abcg5 double knockout mice (dko) the present study investigated the differential contribution of two of the most relevant transporters, namely ATP-binding cassette sub-family G member 5 and 8 (ABCG5/G8) and scavenger receptor class B type I (SR-BI) to sterol metabolism and RCT. Plasma cholesterol levels increased in the following order, mainly due to differences in HDL: Abcg5 ko < wild-type < Sr-bI/Abcg5 dko < Sr-bI ko. Liver cholesterol content was elevated in Sr-bI ko only ($p<0.05$). In Sr-bI/Abcg5 dko plasma plant sterols were highest, while hepatic plant sterols were lower compared with Abcg5 ko ($p<0.05$). Under baseline conditions, biliary cholesterol secretion rates decreased in the following order: wild-type > Sr-bI ko (-16%) > Abcg5 ko (-75%) > Sr-bI/Abcg5 dko (-94%), all at least $p<0.05$, while biliary bile acid secretion did not differ between groups. However, under supraphysiological conditions, upon infusion with increasing amounts of the bile salt tauroursodeoxycholic acid, Abcg5 became fully rate-limiting for biliary cholesterol secretion. Additional in vivo macrophage-to-feces RCT studies demonstrated an almost 50% decrease in overall RCT in Sr-bI/Abcg5 dko compared with Abcg5 ko mice ($p<0.01$).
These data demonstrate that (i) SR-BI contributes to ABCG5/8-independent biliary cholesterol secretion under basal conditions, (ii) biliary cholesterol mass secretion under maximal bile salt-stimulated conditions is fully dependent on ABCG5/8, and (iii) Sr-bI contributes to macrophage-to-feces RCT independent of Abcg5/g8.

Introduction

Sterol elimination from the body can be accomplished by the liver via biliary secretion [1], and by the intestine via modulation of absorption rates as well as by transintestinal cholesterol efflux [2, 3]. By mediating the excretion of cholesterol, bile acids and plant sterols the biliary secretion pathway has relevant implications for major disease complexes such as gallstone disease [4, 5] and atherosclerotic cardiovascular disease (CVD) [6-8]. In the case of CVD, biliary secretion is a critical step for reverse cholesterol transport (RCT) [6, 9]. RCT comprises the transport of cholesterol from macrophage foam cells within atherosclerotic lesions on high density lipoproteins (HDL) to the liver for excretion into bile and via the feces out of the body [8].

Several transporters localized to the bile canaliculus, the apical plasma membrane of hepatocytes, play essential roles in the biliary secretion process [1]. ATP-binding cassette transporter B4 (ABCB4) is required for the biliary output of phospholipids and is key for the formation of mixed micelles, which are necessary for the efficient biliary excretion of sterols [1, 6]. This is illustrated by Abcb4 ko mice having almost unmeasurable biliary cholesterol secretion rates [10]. Another important positive regulator of biliary cholesterol secretion are bile acids. Biliary bile acid (BA) secretion is mediated by ABCB11 [11, 12], and stimulating biliary BA output directly translates into increased biliary cholesterol secretion rates [13]. ATPase class I type 8B member 1 preserves the rigid structure of the outer canalicular leaflet by inward flipping of phospatidylserine making it more resistent to detergents [14]. The majority of cholesterol is secreted via the obligate heterodimeric transport proteins ATP-binding cassette sub-family G member 5 and 8 (ABCG5/G8), initially identified as the molecular defect in sitosterolemia [15, 16]. Recently Niemann-Pick type C protein 2 has been shown to act as a positive regulator of ABCG5/G8-dependent secretion [17]. Absence of Abcg5 and/or Abcg8 in mice results in plant sterol accumulation and an approximately 75% reduction in biliary cholesterol output [18-20]. However, still 25% of the total biliary cholesterol secretion occurs independent of Abcg5/g8.

The proteins and mechanisms contributing to this Abcg5/g8-independent secretion are not well understood. We recently identified scavenger receptor class B type I (SR-BI) as a protein that localizes to the bile canaliculus [20]. When overexpressed, SR-BI causes a substantial increase in biliary cholesterol content [21] and also in biliary cholesterol secretion rates [20, 22], an effect that was independent of Abcg5/g8 [20]. Notably, hepatic SR-BI overexpression was able to restore the substantially decreased biliary cholesterol secretion rates of Abcg5-deficient mice to levels of wild-type controls [20]. Therefore, SR-BI conceivably represents a prime candidate responsible for the Abcg5/g8-independent part of biliary cholesterol secretion.

The present study tested this hypothesis by generating Abcg5/Sr-bI double deficient mice. In depth characterization of this novel double knockout (dko) mouse model revealed that, (i) under steady-state conditions SR-BI significantly contributes to the Abcg5/g8-independent part of biliary cholesterol secretion, (ii) SR-BI is involved in

the cellular uptake of plant sterols, (iii) under bile acid-stimulated conditions Abcg5/g8 are rate-limiting for the biliary cholesterol secretion process, and (iv) absence of Sr-bI on the Abcg5 ko background translates also into a substantial decrease in in vivo RCT.

Experimental procedures

Animals
Sr-bI/Abcg5 dko mice were generated by crossing Sr-bI ko mice (Jackson Laboratories, Bar Harbor, ME, USA) with Abcg5 kos [20]. Littermate controls were from the breeding colony. Animals were housed in temperature-controlled rooms (21°C) with alternating 12 hour periods of light and dark and ad libitum access to water and chow diet (Arie Blok, Woerden, The Netherlands). Animal experiments were performed in accordance with national laws. The responsible ethics committee of the University of Groningen approved all protocols.

Analysis of plasma lipids and lipoproteins
Blood was obtained by cardiac puncture under anesthesia following a 4 h fast. Aliquots of plasma were stored at -80°C until analysis. Plasma phospholipids and triglycerides were measured enzymatically (Roche Diagnostics, Mannheim, Germany and Diasys, Holzheim, Germany, respectively), cholesterol and plant sterols/stanols were measured by gas chromatography–mass spectrometry (GC-MS) as previously described [20]. Pooled plasma samples were subjected to fast protein liquid chromatography (FPLC) gel filtration using a superose 6 column (GE Healthcare, Little Chalfont, UK) as described [23].

Analysis of liver lipid composition
Liver tissue was homogenized, lipids extracted according to the general procedure of Bligh and Dyer and lipids were redissolved in water containing 2% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) [24]. Phospholipid content was determined as published [24], sterols were analyzed by GC-MS as detailed above.

Bile collection and composition analysis
The galbladder was cannulated under anesthesia, bile was collected for 30 min and production was determined gravimetrically [24]. Body temperature was maintained using a humidified incubator [24]. Biliary cholesterol, phospholipid, bile salt, plant sterol/stanol concentrations were determined and the respective biliary secretion rates calculated [24].

Bile acid infusion experiments
The bile acid infusion experiment was performed as described [13]. After gallbladder cannulation and two basal bile samples the mice were continuously infused with

tauroursodeoxycholate (TUDCA; Calbiochem/Merck Biosciences, Darmstadt, Germany) in PBS via the jugular vein. TUDCA infusion rates were increased in a stepwise manner: 150, 300, 450 and 600 nmol/min. Bile samples were collected at 15-minute intervals for 210 min (three samples per infusion step).

Analysis of hepatic gene expression by real-time PCR
Real-time quantitative PCR was performed as published on a 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) [20]. mRNA expression levels were calculated relative to the average of the houskeeping gene cyclophilin and further nomalized to the expression levels of the respective controls.

Electron microscopy
Mice were anesthetized and perfused with 5 ml fixation buffer (4% paraformaldehyde, 2% glutaraldehyde, pH 7.4) [20]. Ultrathin sections (70-100 nm) were prepared on the basis of Toluidine blue stained 1 μm sections, contrast-stained with 2% uranyl acetate and lead citrate according to routine procedures, and examined using a Philips CM100 electron microscope operating at 80 kV.

In vivo macrophage-to-feces reverse cholesterol transport studies
Experiments were essentially performed as described before using thioglycollate-elicited primary peritoneal macrophages from C57BL/6J donor mice loaded in vitro with 50 μg/ml acetylated LDL and 3 μCi/ml [3H]cholesterol (Perkin Elmer, Boston, MA, USA) for 24 hours to become foam cells [25]. These were injected intraperitoneally into individually housed recipient mice. Counts in plasma (6, 24, 48h) were assessed directly by liquid scintillation counting (Packard 1600CA Tri-carb, Packard, Meriden, CT, USA), tracer uptake into the liver was determined at 48h following solubilization of the tissue (Solvable, Packard, Meriden, CT, USA) as described [26]. Feces were collected for 48h, then dried, weighed and ground. After incubation in Solvable counts were determined by liquid scintillation counting and related to the total amount of feces produced over the 48 h experimental period. All obtained counts were expressed relative to the administered tracer dose.

Statistics
Statistical analyses were performed using GraphPad Prism (GraphPad software, San Diego, CA, USA). Data are presented as means ± SEM. Statistical differences between two groups were assessed using the Mann-Whitney U-test. When comparing multiple groups, statistical analysis was performed using ANOVA followed by a Bonferroni post-test or Repeated Measures ANOVA followed by a Bonferroni post-test (Figure 4). The coupling of cholesterol secretion to bile acids and phospholipids (Figure 5) was analyzed with linear regression and slopes were compared. Statistical significance for all comparisons was assigned at $p<0.05$.

Results

In the absence of Sr-bI plant sterols accumulate in plasma, preferentially in HDL
Sr-bI/Abcg5 dko mice were characterized in comparison to wild-type controls and the respective Sr-bI and Abcg5 single ko mice. Plasma total cholesterol levels were increased in the Sr-bI ko ($p<0.05$) and significantly decreased in the Abcg5 ko model ($p<0.05$), but were unchanged in Sr-bI/Abcg5 dko mice compared to wild-type controls (Figure 1A). However, FPLC profiles showed major changes in the lipoprotein profile attributable to altered Sr-bI as well as Abcg5 expression (Figure 1B). While Sr-bI ko mice had a higher and broader HDL peak with a clear shift towards larger particles (Figure 1B), Abcg5 ko mice exhibited a lower HDL peak with no apparent shift in particle size compared with controls. The Sr-bI/Abcg5 dko mice showed a combination of these two effects, the HDL peak was lower than in Sr-bI ko mice but still had an appreciable shift towards larger particles (Figure 1B). Plasma plant sterol levels increased gradually from wild-type over Sr-bI ko and Abcg5 ko mice towards the dko (Figure 1C). While the increase in plasma plant sterols in Abcg5 ko mice is consistent with the intestinal plant sterol hyperabsorption already described in this mouse model [18], the further increase in the absence of Sr-bI indicates that Sr-bI might be involved in cellular plant sterol uptake. When the plant sterol content of isolated lipoprotein fractions was investigated, we noticed that these are preferentially contained within HDL (Figure 1D). Plasma plant stanols largely followed the pattern observed for plant sterols (Figures 1E and F). A detailed overview of alterations in specific individual sterols in plasma in the different models is presented in table 1.

Hepatic plant sterol content is decreased in the absence of Sr-bI
Body weight of Abcg5 ko mice was decreased ($p<0.05$, table 1). Livers of the different models showed no apparent differences in size or gross morphology (Figure 2A). However, liver weight as percentage of body weight was lower in Sr-bI ko mice and higher in Abcg5 ko and Sr-bI/Abcg5 dko mice ($p<0.05$, table 1). As examined by electron microscopy, there were no apparent differences between the groups on the ultrastructural level, in particular the morphology of bile canaliculi was not altered and there was no apparent indication of cholestasis in all of the models (Figure 2B). Liver total cholesterol content was increased in Sr-bI ko mice compared to the other models (Figure 2C, $p<0.05$). Thereby the hepatic content of cholesterol esters was highest in Sr-bI ko ($p<0.05$), while the Sr-bI/Abcg5 dko mice had decreased hepatic cholesterol esters compared to wild-type mice (Figure 2D, $p<0.05$). Liver free cholesterol content was increased in Sr-bI ko mice only ($p<0.05$, data not shown). There were no differences in hepatic phospholipid content (data not shown). Total hepatic plant sterol (Figure 2E) and stanol (Figure 2F) contents were rather low in mice with intact Abcg5, whereas the Abcg5 ko model exhibited a substantial increase in hepatic plant sterols as well as stanols ($p<0.05$). However, in Sr-bI/Abcg5 dko mice hepatic plant sterols and stanols were significantly lower as in the Abcg5 single ko model ($p<0.05$, Figure 2E and F) indicating a potential involvement of SR-BI in the uptake

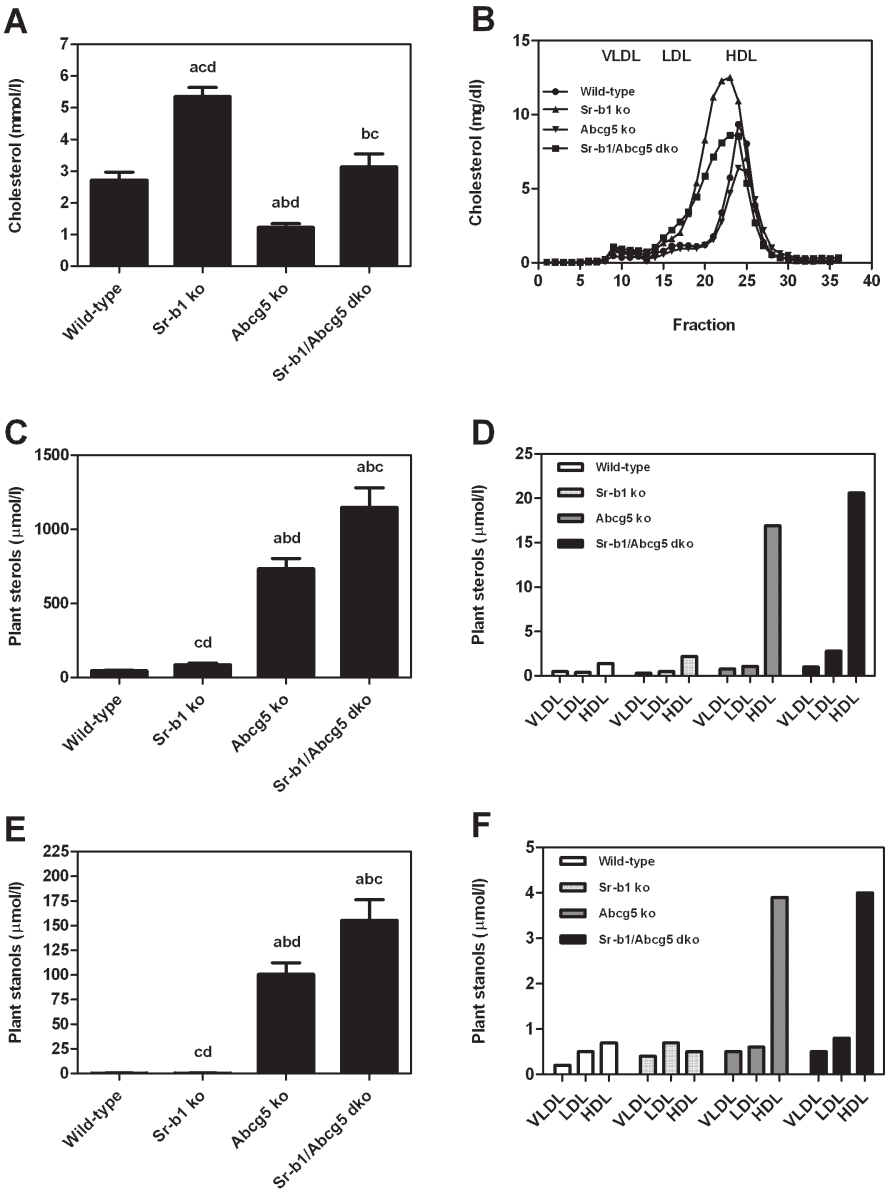

Figure 1: Differential impact of Sr-bI and Abcg5 expression on levels and distribution of plasma sterols. Wild-type, Sr-bI ko, Abcg5 ko and Sr-bI/Abcg5 dko mice were fasted for 4 hours and a blood sample was taken. (A) Plasma total cholesterol levels, (B) FPLC cholesterol profiles of pooled plasma samples, (C) plasma plant sterol levels, (D) distribution of plant sterols over the VLDL, LDL and HDL fractions, (E) plasma plant stanol levels, (F) distribution of plant stanols over the VLDL, LDL and HDL fractions. Data in A, C and E are presented as means ± SEM. n = 6 for each group. a indicates statistically significant differences from wild-type, b from Sr-bI ko, c from Abcg5 ko and d from Sr-bI/Abcg5 dko mice (at least $p<0.05$).

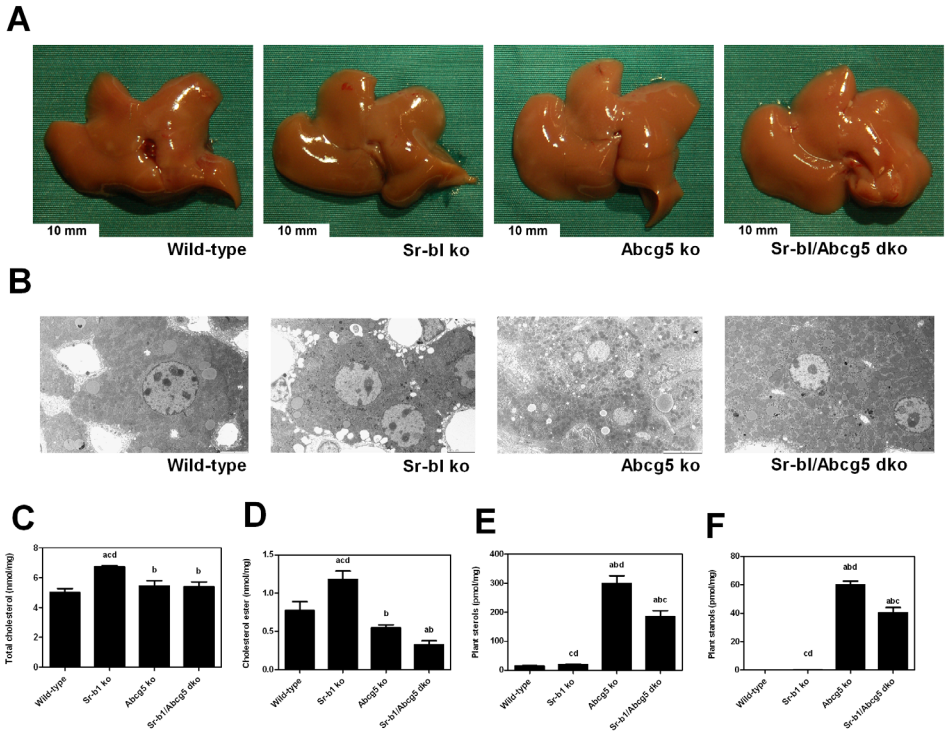

Figure 2: Effect of Sr-bI and Abcg5 expression on liver morphology and hepatic sterol content. (A) Livers from wild-type, Sr-bI ko, Abcg5 ko and Sr-bI/Abcg5 dko mice show no apparent differences in weight, size or gross morphology. (B) Representative EM pictures of livers from wild-type, Sr-bI ko, Abcg5 ko and Sr-bI/Abcg5 dko mice showing no alterations in ultrastructural morphology. Hepatic contents of (C) total cholesterol, (D) free cholesterol, (E) plant sterols, (F) plant stanols. Data in C, D, E and F are presented as means ± SEM. n = 6 for each group. a indicates statistically significant differences from wild-type, b from Sr-bI ko, c from Abcg5 ko and d from Sr-bI/Abcg5 dko mice (at least $p<0.05$).

of plant sterols and stanols. Changes in total as well as individual hepatic sterols are summarized in table 1.

Biliary cholesterol secretion is decreased in Sr-bI/Abcg5 dko mice under steady-state conditions

Next we performed continuous bile cannulation experiments in the four different mouse models. Bile flow was increased in Sr-bI/Abcg5 dko compared with wild-type and Sr-bI ko mice ($p<0.05$, Figure 3A). Phospholipid and bile acid secretion rates were both unchanged among the models (Figure 3B and C). Biliary cholesterol secretion was significantly lower in Abcg5 ko mice consistent with previous reports [13, 20], however, was even further decreased in Sr-bI/Abcg5 dko mice ($p<0.05$, Figure 3D). These data indicate that Sr-bI contributes significantly to the Abcg5-independent part of biliary cholesterol secretion. Plant sterol secretion rates were unchanged

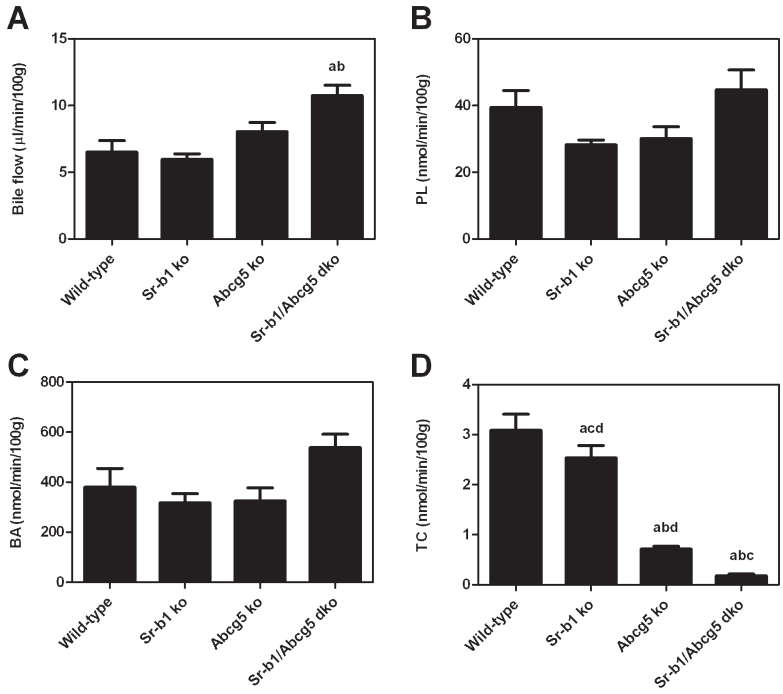

Figure 3: Differential impact of Sr-bI and Abcg5 on biliary sterol secretion under steady-state conditions. Continuous bile cannulation experiments for 30 min were performed in wild-type, Sr-bI ko, Abcg5 ko and Sr-bI/Abcg5 dko mice as detailed in materials and methods. (A) Bile flow, biliary secretion rates of (B) phospholipids, (C) bile acids, and (D) cholesterol. Data are presented as means ± SEM. n = 6 for each group. a indicates statistically significant differences from wild-type, b from Sr-bI ko, c from Abcg5 ko and d from Sr-bI/Abcg5 dko mice (at least $p<0.05$).

among the four models (table 1), however, plant stanol secretion was higher in Abcg5 ko as well as Sr-bI/Abcg5 dko mice (table 1). Biliary secretion rates for individual sterols are also given in table 1. Hepatic mRNA expression of Mdr2 and Bsep were unchanged among the four models (table 2). Abcg5 mRNA expression was unaltered in Sr-bI ko mice. Sr-bI mRNA expression was increased by 48% in Abcg5 ko mice ($p<0.05$, table 2), however, Sr-bI protein expression was decreased (supplemental figure I). Hepatic expression of Srebp2 and its target genes Ldlr and Hmgcoar was decreased in the absence of Abcg5 (table 2). Cyp7a1 expression was elevated in Sr-bI and Abcg5 ko mice, while Cyp8b1 was higher in Abcg5 ko and decreased in the models lacking Sr-bI (table 2). However, these mRNA expression changes in bile acid synthesis enzymes did not translate into altered plasma bile acid levels, bile acid pool size, fecal bile acid excretion or composition of the bile acid pool in the four models studied (supplemental table I). A detailed analysis of intestinal gene expression is provided in supplemental table II.

Table 1: Body weight, liver/body weight ratio and sterol species in plasma, liver and bile of wild-type, Sr-bI knockout, Abcg5 knockout and Sr-bI/Abcg5 double knockout mice.

	Wild-type	Sr-bI ko	Abcg5 ko	Sr-bI/Abcg5 dko
Body weight (g)	31.0 ± 0.9	30.5 ± 1.8	25.2 ± 1.2	26.8 ± 1.0
Liver/body weight ratio	0.048 ± 0.001	0.039 ± 0.002^{acd}	0.060 ± 0.003^{ab}	0.056 ± 0.001^{ab}
Sterol species in plasma				
Cholesterol (mmol/l)	2.56 ± 0.15	5.32 ± 0.35^{acd}	1.22 ± 0.13^{abd}	3.13 ± 0.40^{bc}
Brassicasterol (µmol/l)	0.44 ± 0.04	0.82 ± 0.11^{cd}	6.23 ± 0.34^{abd}	19.64 ± 2.16^{abc}
Campesterol (µmol/l)	31.78 ± 2.40	62.43 ± 9.09^{cd}	234.92 ± 20.29^{abd}	364.83 ± 37.93^{abc}
Campestanol (µmol/l)	0.46 ± 0.04	0.60 ± 0.11^{cd}	64.05 ± 7.56^{abd}	93.41 ± 12.07^{abc}
Stigmasterol (µmol/l)	0.10 ± 0.00	0.14 ± 0.03^{cd}	9.16 ± 0.43^{abd}	23.05 ± 2.67^{abc}
β-Sitosterol (µmol/l)	9.71 ± 0.68	21.21 ± 2.88^{cd}	484.40 ± 47.83^{abd}	740.37 ± 90.08^{abc}
Sitostanol (µmol/l)	0.10 ± 0.00	0.14 ± 0.04^{cd}	36.63 ± 4.05^{abd}	61.86 ± 8.79^{abc}
Sterol species in liver				
Cholesterol (nmol/mg liver)	1.07 ± 0.11	1.70 ± 0.10^{acd}	0.64 ± 0.08^{ab}	0.75 ± 0.05^{b}
Brassicasterol (pmol/mg liver)	0.35 ± 0.09	0.37 ± 0.10^{cd}	6.10 ± 0.50^{ab}	6.00 ± 0.74^{ab}
Campesterol (pmol/mg liver)	11.58 ± 1.19	15.66 ± 1.55^{cd}	108.18 ± 11.01^{abd}	71.47 ± 7.11^{abc}
Campestanol (pmol/mg liver)	N.D.	0.22 ± 0.14^{cd}	38.06 ± 1.38^{abd}	25.51 ± 2.08^{abc}
Stigmasterol (pmol/mg liver)	0.04 ± 0.03	0.09 ± 0.5^{cd}	9.18 ± 0.70^{abd}	5.98 ± 0.59^{abc}
β-Sitosterol (pmol/mg liver)	2.55 ± 0.27	3.10 ± 0.16^{cd}	174.81 ± 16.22^{abd}	101.82 ± 12.18^{abc}
Sitostanol (pmol/mg liver)	N.D.	N.D.	22.03 ± 1.23^{abd}	14.96 ± 1.60^{abc}
Total sterols & stanols (pmol/mg liver)	1089.02 ± 108.30	1720.48 ± 97.30^{acd}	1000.96 ± 98.81^{b}	975.41 ± 62.09^{b}
Sterol species in bile				
Brassicasterol (pmol/min/100g bw)	9.21 ± 1.03	5.67 ± 0.30	7.49 ± 1.01	9.10 ± 1.52
Campesterol (pmol/min/100g bw)	126.41 ± 16.11	112.78 ± 12.85	95.44 ± 13.81	83.59 ± 5.77
Campestanol (pmol/min/100g bw)	7.40 ± 1.25	7.39 ± 0.58^{cd}	20.51 ± 2.16^{ab}	15.72 ± 0.79^{ab}
Stigmasterol (pmol/min/100g bw)	1.91 ± 0.11	1.46 ± 0.09^{cd}	5.25 ± 0.51^{abd}	2.69 ± 0.19^{bc}
β-Sitosterol (pmol/min/100g bw)	54.77 ± 6.64	40.12 ± 3.15^{c}	97.06 ± 12.37^{ab}	71.97 ± 5.68
Sitostanol (pmol/min/100g bw)	1.91 ± 0.11	1.46 ± 0.09^{cd}	5.53 ± 0.30^{abd}	3.74 ± 0.62^{abc}

Mice were fasted for 4 h. Blood was collected by cardiac puncture under anesthesia. Livers were removed, weighed and stored at -80ºC until analysis. Continuous bile cannulations were performed for 30 min. Cholesterol and individual phytosterol concentrations were determined in plasma, liver and bile by GC-MS. Individual phytosterol biliary secretion rates were calculated. Data are presented as means ± SEM. n = 6 for each group. a indicates statistically significant differences from wild-type, b from Sr-bI knockout, c from Abcg5 knockout and d from Sr-bI/Abcg5 double knockout mice (at least $p<0.05$). N.D. is not detected. bw, body weight.

Abcg5 is rate-limiting for biliary cholesterol secretion under bile acid-stimulated conditions

Since biliary cholesterol secretion is lower in Sr-bI/Abcg5 dko mice under steady-state conditions, we next aimed to determine the effects of maximally stimulating biliary cholesterol secretion in a bile acid infusion experiment. TUDCA was infused in a stepwise manner from 150 up to 600 nmol/min. Upon TUDCA infusion bile

Table 2: Hepatic mRNA expression in wild-type, Sr-bI knockout, Abcg5 knockout and Sr-bI/Abcg5 double knockout mice.

	Wild-type	Sr-bI ko	Abcg5 ko	Sr-bI/Abcg5 dko
Hepatic mRNA expression				
Sr-bI	1.00 ± 0.05	N.D.	1.48 ± 0.06[a]	N.D.
Ldlr	1.00 ± 0.04	0.96 ± 0.08[c]	0.54 ± 0.03[acd]	0.85 ± 0.04[c]
Abca1	1.00 ± 0.04	1.00 ± 0.05	1.11 ± 0.08	1.02 ± 0.06
Abcg5	1.00 ± 0.10	0.89 ± 0.12	N.D.	N.D.
Abcg8	1.00 ± 0.07	0.72 ± 0.07[cd]	1.09 ± 0.06[b]	1.07 ± 0.11[b]
Npc2	1.00 ± 0.03	1.11 ± 0.05	1.15 ± 0.11	1.19 ± 0.16
Abcb4	1.00 ± 0.06	0.86 ± 0.09	0.93 ± 0.06	0.74 ± 0.05
Abcb11	1.00 ± 0.05	0.86 ± 0.09	1.17 ± 0.06	1.11 ± 0.08
Hmgcoar	1.00 ± 0.15	1.17 ± 0.12[cd]	0.35 ± 0.05[ab]	0.69 ± 0.05[b]
Cyp7a1	1.00 ± 0.22	1.91 ± 0.26	2.66 ± 0.35[a]	2.00 ± 0.28
Cyp8b1	1.00 ± 0.11	0.81 ± 0.14[c]	1.38 ± 0.12[bd]	0.81 ± 0.04[c]
Srebp2	1.00 ± 0.04	0.87 ± 0.04	0.67 ± 0.04[a]	0.77 ± 0.07[a]
Srebp1c	1.00 ± 0.07	1.12 ± 0.14	0.85 ± 0.13[d]	1.36 ± 0.11[c]
Lxra	1.00 ± 0.03	1.15 ± 0.05	0.94 ± 0.03	1.04 ± 0.10
Lxrb	1.00 ± 0.06	0.86 ± 0.02	0.93 ± 0.03	0.84 ± 0.06

Mice were fasted for 4 h, livers were removed, weighed and stored at -80°C until analysis. Individual genes are expressed as a percentage of the houskeeping gene cyclophilin and further nomalized to the expression levels of the respective controls. Data are presented as means ± SEM. n = 6 for each group. a indicates statistically significant differences from wild-type, b from Sr-bI knockout, c from Abcg5 knockout and d from Sr-bI/Abcg5 double knockout mice (at least $p<0.05$). N.D. is not detected.

flow increased by at least 200 percent without differences among the models (data not shown). Biliary bile acid secretion was similarly stimulated in all four models according to the bile acid infusion rate (Figure 4A). Phospholipid secretion also increased upon TUDCA infusion (Figure 4B) with Sr-bI ko mice having the highest secretion rates and Abcg5 the lowest ($p<0.01$ compared to Sr-bI ko). Sr-bI/Abcg5 dko mice had an intermediate response (Figure 4B). In wild-type controls, biliary cholesterol secretion increased in response to TUDCA infusion, while surprisingly Sr-bI ko mice even displayed a trend towards an increased responsiveness (Figure 4C). However, in both models with absent Abcg5 expression, namely Abcg5 ko and Sr-bI/Abcg5 dko mice, there was no appreciable impact of TUDCA infusion on biliary cholesterol secretion. These data strongly indicate that the bile acid-stimulated increase in biliary cholesterol secretion is fully dependent upon Abcg5 expression. Figure 5 summarizes the results of the bile acid infusion experiment by depicting the biliary cholesterol secretion as a function of biliary bile acid (Figure 5A) as well as phospolipid secretion (Figure 5B). On the one hand, these data indicate an enhanced response in biliary cholesterol secretion to increasing biliary bile acid secretion rates

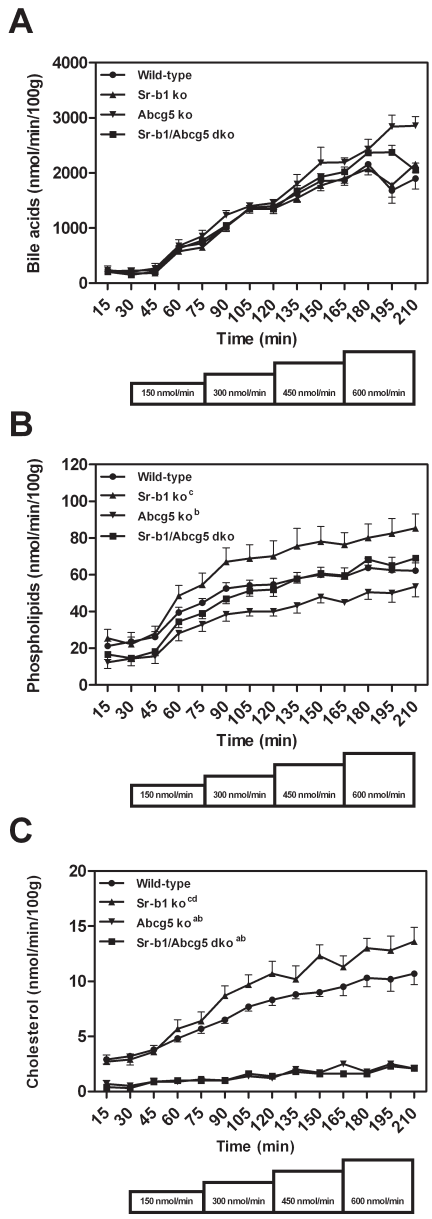

Figure 4: Differential impact of Sr-bI and Abcg5 on biliary sterol secretion under bile acid-stimulated conditions. Continuous bile cannulation experiments were performed in wild-type, Sr-bI ko, Abcg5 ko and Sr-bI/Abcg5 dko mice that were infused with TUDCA. The infusion rate was increased in a stepwise manner from 150 to 600 nmol/min as indicated, bile samples were collected for 15 min each as detailed in materials and methods. Biliary secretion rates of (A) bile acids, (B) phospholipids, and (C) cholesterol in response to the TUDCA infusion. Data are presented as means ± SEM. n = 6 for each group. a indicates statistically significant differences from wild-type, b from Sr-bI ko, c from Abcg5 ko and d from Sr-bI/Abcg5 dko mice (at least $p<0.05$).

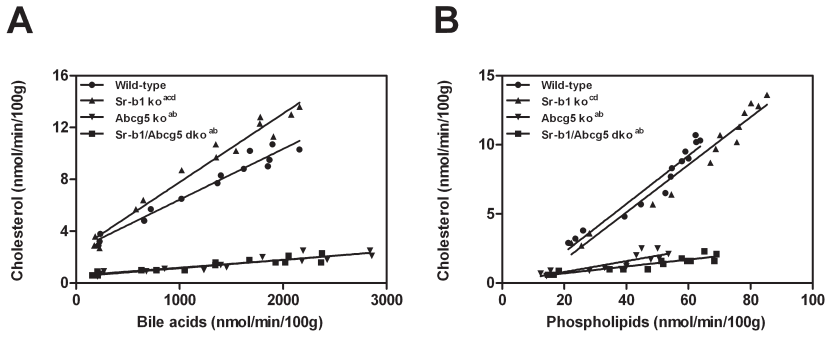

Figure 5: Differential impact of Sr-bI and Abcg5 on the dependency of biliary cholesterol secretion on biliary bile acid as well as phospholipid secretion. The combined data from the TUDCA infusion experiment shown in figure 4 were related to each other to show (A) biliary cholesterol secretion as a function of bile salt secretion and (B) biliary cholesterol secretion as a function of phospholipid secretion. n = 6 for each group. a indicates statistically significant differences from wild-type, b from Sr-bI ko, c from Abcg5 ko and d from Sr-bI/Abcg5 dko mice (at least $p<0.05$).

in the Sr-bI ko model (at least $p<0.05$). On the other hand, the graphs show that biliary cholesterol secretion does not respond to increasing biliary bile acid and phospholipid secretion rates, when Abcg5 is absent ($p<0.001$ compared with wild-types and Sr-bI ko).

Sr-bI contributes to reverse cholesterol transport independent of Abcg5/g8 expression
Macrophage-to-feces RCT has previously been demonstrated to be decreased in Sr-bI ko mice [27]. Biliary cholesterol secretion plays a critical role in RCT [6] Since biliary cholesterol secretion was further significantly decreased in Sr-bI/Abcg5 dko compared to Abcg5 single ko, we next performed a RCT experiment comparing Abcg5 ko with Sr-bI/Abcg5 dko mice to determine the impact of Sr-bI independent of Abcg5. Plasma 3H-cholesterol tracer levels were higher in Sr-bI/Abcg5 dko mice compared to Abcg5 ko ($p<0.05$ at 4h and 24, $p=0.059$ at 48h, Figure 6A). In addition, hepatic 3H-cholesterol tracer recovery at 48h was significantly decreased in Sr-bI/Abcg5 dko mice (Figure 6B). Most importantly, however, there was a substantial reduction in total macrophage-to-feces RCT by almost 50% in Sr-bI/Abcg5 dko mice compared to the Abcg5 single ko model. These data demonstrate that the additional independent impact of SR-BI deficiency on biliary cholesterol secretion in mice with absent Abcg5 expression translates functionally into decreased RCT.

Discussion

The present study primarily investigated the hypothesis that SR-BI contributes to the ABCG5/G8-independent part of biliary cholesterol secretion. To address this question, a novel dko mouse model for Abcg5 and Sr-bI was generated and characterized.

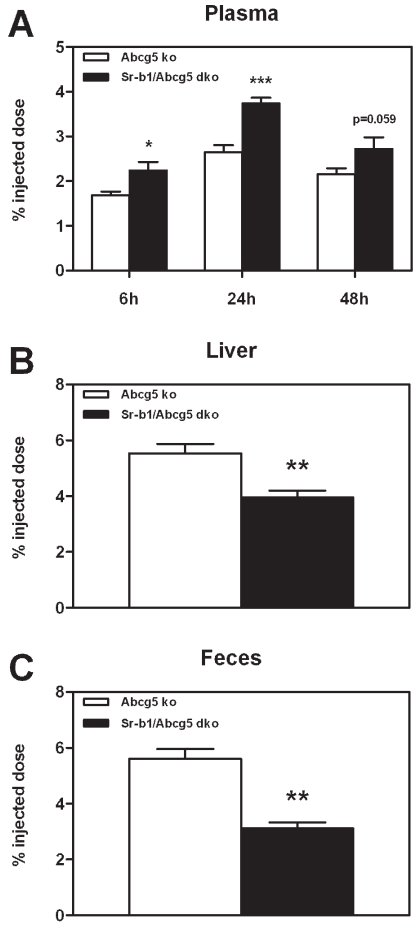

Figure 6: Deficiency of Sr-bI decreases in vivo macrophage-to-feces reverse cholesterol transport independent of Abcg5 expression. An in vivo macrophage-to-feces reverse cholesterol transport experiment was performed in Abcg5 ko and Sr-bI/Abcg5 dko mice following intraperitoneal injections with 3H-cholesterol-loaded primary mouse macrophage foam cells as detailed in materials and methods. (A) 3H-cholesterol tracer appearance in plasma 6, 24 and 48 h after macrophage administration. (B) 3H-cholesterol tracer recovery within liver 48 h after macrophage injection. (C) 3H-cholesterol tracer appearance in feces collected continuously from 0 to 48 h after macrophage administration. Data are presented as means ± SEM. n = 8 for each group. Statistically significant differences from Abcg5 ko mice are indicated as *p<0.05, **p<0.01, ***p<0.001.

Our data demonstrate that (i) SR-BI contributes to the ABCG5-independent part of biliary cholesterol secretion under steady-state conditions, (ii) SR-BI is involved in the cellular uptake of plant sterols, (iii) the increase in the bile acid-stimulated secretion of cholesterol into the bile is fully dependent on ABCG5 without further contribution by SR-BI, and (iv) consistent with the effects on biliary cholesterol secretion, Sr-bI deficiency in Abcg5 ko mice substantially decreases in vivo RCT.

SR-BI has previously been characterized as the selective uptake receptor for HDL cholesterol into liver and steroidogenic tissues [21, 28]. Consequently, deficiency in SR-BI is associated with higher HDL cholesterol levels in plasma and a shift towards larger HDL particles [29]. The results of our present study demonstrate that plant sterols also accumulate in plasma in the absence of Sr-bI suggesting that in addition to cholesterol uptake, Sr-bI is important for cellular uptake of plant sterols and stanols. Consistent with this conclusion is the significant decrease in hepatic plant sterol and stanol content when Sr-bI is not expressed.

Regarding biliary cholesterol secretion, two independent reports thus far suggested a slight but consistent decrease in biliary cholesterol secretion rates in Sr-bI ko mice [22, 30]. Overall, these changes translated into a decrease in RCT in the Sr-bI ko model [27]. RCT is a process that comprises the movement of cholesterol from macrophage foam cells within atherosclerotic lesions via HDL to the liver for final excretion into bile and feces and is thereby highly relevant for the prevention as well as potentially the treatment of atherosclerotic CVD [31]. Hepatic overexpression of SR-BI on the other hand decreases plasma HDL cholesterol levels and increases hepatic cholesterol content due to enhanced mass cholesterol uptake from HDL [22, 23, 32]. In addition, hepatic SR-BI overexpression results in a significant increase in bile cholesterol content [21] as well as biliary cholesterol secretion rates [20, 22]. These changes translate directly into a significant increase in in vivo RCT [27]. Our results indicate that the impact of SR-BI on RCT is apparently independent of ABCG5, since a substantial reduction in completed RCT was noted in Sr-bI/Abcg5 dko mice compared with the Abcg5 single ko model. These data suggest that, although Abcg5/g8 transport the bulk of cholesterol into bile, Sr-bI has differential functionalities independent of Abcg5/g8 that relate to biliary cholesterol secretion from a pool with a high relevance for RCT.

Importantly, we previously demonstrated that adenovirus-mediated overexpression of Sr-bI increases biliary cholesterol secretion independent of Abcg5 expression and was able to restore the reduced biliary cholesterol secretion rates of Abcg5 ko mice to the levels of normal wild-type controls [20]. Our present study on the other hand demonstrates that under steady-state conditions there is a further significant decrease in biliary cholesterol secretion when Sr-bI is knocked out in Abcg5-deficient mice. Interestingly though, these differences are no longer apparent under bile acid-stimulated conditions. On the contrary, Sr-bI ko mice on a wild-type background even exhibit a trend towards increased biliary cholesterol secretion. The mechanism underlying this observation is currently unclear and further experimentation would be required to delineate the functional interplay between intrahepatic cholesterol pools and basolaterally and apically localized Sr-bI. In the dko mice on the other hand Abcg5 is clearly rate-limiting with its absence preventing any significant increase in biliary cholesterol secretion in response to bile acid infusion.

ABCG5 and ABCG8 are ABC half transporters that form an obligate heterodimer for full functionality [16, 33]. Mutations in either ABCG5 or ABCG8 have been characterized as the genetic substrate for sitosterolemia, a disease that is characterized by an

accumulation of plant sterols within the body due to increased intestinal absorption and diminished biliary excretion in the absence of functional ABCG5/G8 expression [34]. In addition, patients with sitosterolemia display accelerated atherosclerotic lesion formation [35]. Interestingly, certain mutations in ABCG5/G8 have also reproducibly been associated with genetically conferred susceptibility to cholesterol gallstone formation demonstrating that understanding the functionality of this heterodimeric transporter pair is also of prime relevance for gallstone disease [36-38].
In summary, the characterization of Sr-bI/Abcg5 double-deficient mice in the present study demonstrates that under steady-state but not under bile acid-stimulated conditions SR-BI contributes significantly to the Abcg5/g8-independent part of biliary cholesterol secretion. Pathophysiologically, an additional deficiency in Sr-bI also translates into a substantial decrease in in vivo RCT in Abcg5 ko mice. However, when biliary cholesterol output was maximally stimulated by bile acid infusion, Abcg5/g8 were rate-limiting stressing the important role of this transporter heterodimer in mass cholesterol secretion into bile. These data add to current concepts on the differential roles and functionalities of SR-BI and ABCG5/G8 in the biliary sterol secretion process, a key mechanism relevant for atherosclerotic CVD as well as gallstone disease.

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Supplemental material chapter 2

Supplemental experimental procedures

Animals
Sr-bI knockout mice were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). The Abcg5 knockout model has been described previously [1]. Sr-bI/Abcg5 double knockout mice were generated by crossing the respective single knockouts. Littermate controls were also derived from the breeding colony. All animals were housed in temperature controlled rooms (21°C) with alternating 12 hour periods of light and dark and ad libitum access to water and mouse chow diet (Arie Blok, Woerden, The Netherlands). Animal experiments were performed in accordance with national laws. The responsible ethics committee of the University of Groningen approved all protocols.

Analysis of plasma lipids and lipoproteins
Blood was obtained by cardiac puncture under anesthesia following a 4 h fast. Aliquots of plasma were stored at -80°C until analysis. Plasma phospholipids and triglycerides were measured enzymatically using comercially available reagents (Roche Diagnostics, Mannheim, Germany and Diasys, Holzheim, Germany, respectively), cholesterol was measured by gas chromatography–mass spectrometry (GC-MS) as previously described [2]. Pooled plasma samples were subjected to fast protein liquid chromatography (FPLC) gel filtration using a superose 6 column (GE Healthcare, Little Chalfont, UK) as described [3]. Total cholesterol was measured in individual fractions as detailed above. VLDL, LDL and HDL fractions were pooled and cholesterol and plant sterol concentrations were determined by by GC-MS.

Analysis of liver lipid composition
Liver tissue was homogenized and total cholesterol and free cholesterol were measured using commercially available reagents (Roche Diagnostics, Mannheim, Germany and Diasys, Holzheim, Germany) after extracting lipids according to the general procedure of Bligh and Dyer and redissolving the lipids in water containing 2% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) [4]. Phospholipid content was determined after lipid extraction as described previously [4], and sterols were analyzed by GC-MS as detailed above.

Bile collection and composition analysis
The galbladder was cannulated under hypnorm (fentanyl/fluanisone; 1 ml/kg bodyweight) and diazepam (10 mg/kg bodyweight) anesthesia, bile was collected for 30 min and production was determined gravimetrically [4]. Body temperature was maintained by using a humidified incubator [4]. Biliary cholesterol, phospholipid, bile salt, plant sterol as well as stanol concentrations were determined and the respective biliary secretion rates were calculated [4].

Bile acid infusion experiments
The bile acid infusion experiment was performed as described before [5]. The gallbladder was cannulated as detailed above. After two basal bile samples the mice were continuously infused with tauroursodeoxycholate (TUDCA; Calbiochem/Merck Biosciences, Darmstadt, Germany) in PBS via the jugular vein [5]. The TUDCA infusion rates were increased in a stepwise manner: 150, 300, 450 and 600 nmol/min [5]. Bile samples were collected at 15-minute intervals for 210 min (three samples per infusion step).

Analysis of gene expression by real-time PCR
Total RNA from mouse livers, proximal intestine and distal intestine was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantified with a NanoDrop 2000c UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA) as described before [6]. cDNA synthesis was performed from 1 μg of total RNA using reagents from Invitrogen (Carlsbad, CA, USA). Real-time quantitative PCR was performed on a 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) [1]. PCR primers and fluorogenic probes were designed with Primer Express Software (Applied Biosystems, Foster City, CA, USA) and synthesized by Eurogentec (Seraing, Belgium). mRNA expression levels were calculated relative to the average of the houskeeping gene cyclophilin and further nomalized to the expression levels of the respective controls.

Electron microscopy
Mice were anesthetized (Isoflurane in combination with 30-40% O¬2) and perfused with 5 ml fixation buffer (4% paraformaldehyde, 2% glutaraldehyde, pH 7.4) by injection into the heart [1]. Fixed organs were dissected out. For morphological examination, tissue blocks were cut into small pieces of about 1 mm3. After immersion fixation in 2% glutaraldehyde in 0.1M phosphate buffer, the specimens were postfixed in 1% OsO4 supplemented with 1% K4Fe(CN)6 for 1 h, carefully rinsed in cacodylate buffer, and then dehydrated in a graded ethanol series followed by propylene oxide. Blocks were then infiltrated using a mixture of 1:1 propylene oxide and epon low viscosity resin for 1 h and pure resin overnight. Polymerisation took place at 70 °C after exsiccation in a vacuum. Ultrathin sections (70-100 nm) were prepared on the basis of Toluidine blue stained 1 μm sections, contrast-stained with 2% uranyl acetate in 70% methanol and lead citrate according to routine procedures, and examined using a Philips CM100 electron microscope operating at 80 kV.

In vivo macrophage-to-feces reverse cholesterol transport studies
Experiments were performed as described before [7]. Wild-type C57BL/6J donor mice were injected intraperitoneally with 0.5 ml of 10% thioglycollate (Becton Dickenson, Le Point de Claix, France). On day 4 after injection, peritoneal macrophages were harvested as described [8]. Macrophages were plated in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (HyClone, Logan, UT,

USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). After 4 hours at 37°C under 5% CO2 humidified air nonadherent cells were removed by washing twice with PBS, followed by loading of the macrophages with 50 μg/ml acetylated LDL and 3 μCi/ml [3H]cholesterol (Perkin Elmer, Boston, MA, USA) for 24 hours. Macrophages were washed twice with PBS and equilibrated for 18 h in RPMI 1640 medium containing 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and 2% BSA (Sigma, St. Louis, MO, USA). The macrophages were harvested, resuspended in RPMI 1640 medium and immediately injected intraperitoneally into individually housed recipient mice. Plasma was collected 6 and 24 h after macrophage foam cell injection by retroorbital puncture and for the final blood draw after 48 h by heart puncture. After the experimental period, livers were harvested, snapfrozen in liquid nitrogen, and stored at -80 °C until analysis. Feces were collected continuously for 48 hours after macrophage foam cell injection. Plasma counts were assessed directly by liquid scintillation counting (Packard 1600CA Tri-carb, Packard, Meriden, CT, USA). Uptake of tracer into the liver was determined following solubilization of the tissue (Solvable, Packard, Meriden, CT, USA) as previously described [9]. Counts recovered from a respective piece of liver were backcalculated to total liver mass. Feces were dried, weighed and ground. After incubation in Solvable counts were determined by liquid scintillation counting and related to the total amount of feces produced over the 48 h experimental period. All obtained counts were expressed relative to the administered tracer dose.

Determination of total bile acids in plasma and bile acid pool size
Total BA in plasma were measured as described before [10]. The bile acid pool size was determined using a previously developed and validated stable isotope dilution technique [11]. In short, 240μg of [13C]cholate in a solution of 0.5% NaHCO3 in phosphate-buffered saline was slowly administered via retroorbital injection. Blood samples were taken before injection and at 12, 24, 36, 48 and 60 h after injection. Plasma was stored at -20°C until analysis. Samples were analyzed by GC-MS and calculations were carried out as described [11].

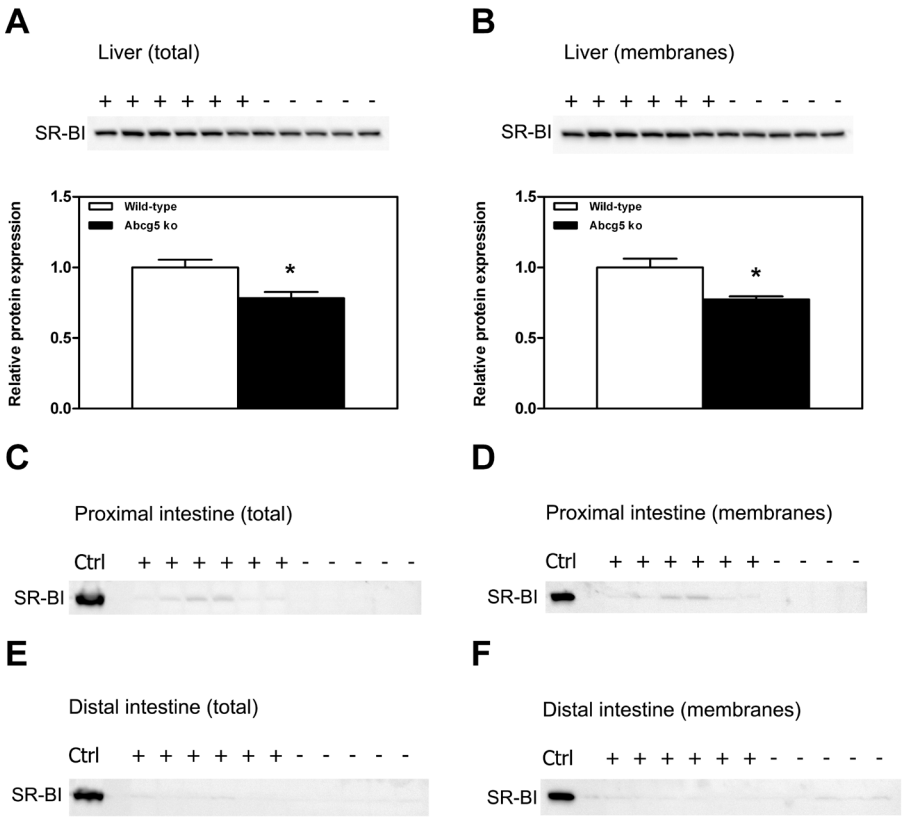
Fecal bile acid analysis
Feces of individually housed animals were collected over a 24-hour period for bile acid composition determination. Fecal samples were dried, weighed and homogenized. Bile acid composition was determined in a 50 mg aliquot of feces by gas liquid chromatography as described [12]. The total amount of bile acids was calculated as the sum of the individually quantified bile acids.

Western blotting
Western blots for SR-BI were carried out on total liver, proximal intestine and distal intestine homogenates as well as on hepatic, proximal intestine and distal intestine membrane fractions prepared essentially as described [7]. Protein concentrations were determined with the bicinchoninic acid (BCA) assay (Pierce Biotechnology,

Inc., Rockford, IL). Equal amounts of protein were resolved by SDS-PAGE electrophoresis and blotted onto nitrocellulose. SR-BI was visualized using a commercially available goat anti-mouse SR-BI antibody (Novus Biologicals, Littleton, CO), followed by the appropriate HRP-conjugated secondary antibody. HRP was detected using chemiluminescence (ECL, GE Healthcare, Piscataway, NJ). Densitometry analysis of the bands was performed on Western blots using ImageJ software (National Institutes of Health, Bethesda, MD). Results were normalized to the relative expression levels of the wild-type controls. Pooled liver samples were used as a positive control for all intestine blots.

Statistics

Statistical analyses were performed using GraphPad Prism (GraphPad software, San Diego, CA, USA). Data are presented as means ± SEM. Statistical differences between two groups were assessed using the Mann-Whitney U-test. When comparing multiple groups, statistical analysis was performed using ANOVA followed by a Bonferroni post-test or Repeated Measures ANOVA followed by a Bonferroni post-test (Figure 4). The coupling of cholesterol secretion to bile acids and phospholipids (Figure 5) was analyzed with linear regression and slopes were compared. Statistical significance for all comparisons was assigned at $p<0.05$.



Supplemental figure I: SR-BI protein expression in liver and intestine of Abcg5 knockout mice. Protein expression of SR-BI in (A) livers, (B) liver membranes, (C) proximal intestine, (D) proximal intestinal membranes, (E) distal intestine and (F) distal intestinal membranes was determined by Western blotting as described in supplemental experimental procedures in wild-type controls and Abcg5 knockout mice. The upper panel of (A) and (B) shows the Western blot and the lower panel its quantification. The SR-BI expression in (C), (D), (E) and (F) is too low to quantify. Data are presented as means ± SEM. n = at least 4 for each group. * indicates statistically significant differences from wild-type mice of at least $p<0.05$. -, Abcg5 ko mice; +, wild-type mice; Ctrl, positive control.

Supplemental table I: Total BA in plasma, BA pool size, total BA in feces and fecal BA composition in wild-type, Sr-bI knockout, Abcg5 knockout and Sr-bI/Abcg5 double knockout mice.

	Wild-type	Sr-bI ko	Abcg5 ko	Sr-bI/Abcg5 dko
Total BA in plasma (µmol/l)	17.12 ± 0.65	18.00 ± 1.07	18.80 ± 0.33	16.97 ± 0.74
BA pool size (µmol/100g bw)	12.36 ± 1.02	14.95 ± 1.92	10.32 ± 0.49	11.40 ± 0.78
Total BA in feces (µmol/24h)	3.05 ± 0.24	2.97 ± 0.34	2.88 ± 0.37	3.73 ± 0.37
BA species in feces				
Allocholic acid (nmol/24h)	107.94 ± 10.91	82.14 ± 7.80^{d}	114.36 ± 22.03^{d}	203.37 ± 35.95^{abc}
Deoxycholic acid (nmol/24h)	1003.55 ± 129.61	636.26 ± 56.73	1119.86 ± 127.00	931.23 ± 193.73
Cholic acid (nmol/24h)	442.87 ± 116.26	500.43 ± 144.27	288.03 ± 61.97	825.20 ± 348.25
Hyodeoxycholic acid (nmol/24h)	75.29 ± 9.49	85.94 ± 21.94	51.85 ± 6.34	46.40 ± 7.02
β-Muricholic acid (nmol/24h)	325.31 ± 29.54	361.95 ± 40.06	311.84 ± 40.20	299.52 ± 32.21
ω-Muricholic acid (nmol/24h)	1096.56 ± 136.43	1348.40 ± 308.44	1014.68 ± 151.50	1436.46 ± 63.14

Total BA in plasma, BA pool size, total BA in feces and fecal BA composition were determined in wild-type, Sr-bI ko, Abcg5 ko and Sr-bI/Abcg5 dko mice. Please see supplemental experimental procedures for details. Data are presented as means ± SEM. n = 6 for each group. a indicates statistically significant differences from wild-type, b from Sr-bI knockout, c from Abcg5 knockout and d from Sr-bI/Abcg5 double knockout mice (at least $p<0.05$). bw, bodyweight.

Supplemental table II: Intestine mRNA expression in wild-type, Sr-bI knockout, Abcg5 knockout and Sr-bI/Abcg5 double knockout mice.

	Wild-type	Sr-bI ko	Abcg5 ko	Sr-bI/Abcg5 dko
Proximal intestine mRNA expression				
Sr-bI	1.00 ± 0.16	N.D.	1.67 ± 0.12[a]	N.D.
Ldlr	1.00 ± 0.09	1.00 ± 0.07	0.96 ± 0.09	0.90 ± 0.04
Abca1	1.00 ± 0.20	1.69 ± 0.11[c]	0.36 ± 0.05[bd]	1.22 ± 0.19[c]
Abcg5	1.00 ± 0.09	1.08 ± 0.19	N.D.	N.D.
Abcg8	1.00 ± 0.11	1.14 ± 0.23[cd]	0.15 ± 0.01[ab]	0.21 ± 0.02[ab]
Npc2	1.00 ± 0.02	1.06 ± 0.04	0.97 ± 0.02	0.96 ± 0.00
Npc1l1	1.00 ± 0.12	0.85 ± 0.04	0.70 ± 0.03	0.74 ± 0.03
Abcb4	N.D.	N.D.	N.D.	N.D.
Asbt	1.00 ± 0.12	1.22 ± 0.20	1.10 ± 0.16	1.13 ± 0.14
Hmgcoar	1.00 ± 0.09	0.90 ± 0.07	0.83 ± 0.05	0.80 ± 0.06
Srebp2	1.00 ± 0.05	0.90 ± 0.03	1.00 ± 0.09	0.88 ± 0.05
Srebp1c	1.00 ± 0.23	2.19 ± 0.76[c]	0.41 ± 0.04[b]	0.85 ± 0.12
Lxra	1.00 ± 0.06	1.14 ± 0.12[cd]	0.65 ± 0.05[ab]	0.81 ± 0.05[b]
Lxrb	1.00 ± 0.06	0.92 ± 0.03	0.73 ± 0.05[a]	0.77 ± 0.00[a]
Distal intestine mRNA expression				
Sr-bI	1.00 ± 0.14	N.D.	0.76 ± 0.06	N.D.
Ldlr	1.00 ± 0.12	0.69 ± 0.06[c]	1.12 ± 0.07[bd]	0.73 ± 0.07[c]
Abca1	1.00 ± 0.21	2.25 ± 0.35[c]	0.67 ± 0.09[bd]	2.46 ± 0.38[ac]
Abcg5	1.00 ± 0.13	1.21 ± 0.22	N.D.	N.D.
Abcg8	1.00 ± 0.15	1.24 ± 0.22[cd]	0.49 ± 0.05[b]	0.67 ± 0.03[b]
Npc2	1.00 ± 0.03	1.09 ± 0.09	0.97 ± 0.02	1.06 ± 0.05
Npc1l1	1.00 ± 0.11	1.18 ± 0.22	0.76 ± 0.07	1.20 ± 0.13
Abcb4	N.D.	N.D.	N.D.	N.D.
Asbt	1.00 ± 0.29	0.67 ± 0.19[c]	2.97 ± 0.38[abd]	0.91 ± 0.36[c]
Hmgcoar	1.00 ± 0.06	0.85 ± 0.04[c]	1.14 ± 0.07[b]	0.76 ± 0.05[ac]
Srebp2	1.00 ± 0.04	0.89 ± 0.08	1.13 ± 0.07[d]	0.87 ± 0.05[c]
Srebp1c	1.00 ± 0.13	1.55 ± 0.29	0.86 ± 0.10	1.19 ± 0.15
Lxra	1.00 ± 0.08	1.09 ± 0.13	0.86 ± 0.08	0.94 ± 0.06
Lxrb	1.00 ± 0.04	1.07 ± 0.04	1.02 ± 0.03	1.21 ± 0.06[a]

Mice were fasted for 4 h, intestines were removed and stored at -80°C until analysis. Individual genes are expressed as a percentage of the houskeeping gene cyclophilin and further nomalized to the expression levels of the respective controls. Data are presented as means ± SEM. n = 6 for each group. a indicates statistically significant differences from wild-type, b from Sr-bI knockout, c from Abcg5 knockout and d from Sr-bI/Abcg5 double knockout mice (at least $p<0.05$). N.D. is not detected.

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Chapter 3

ApoE promotes hepatic selective uptake but not RCT due to increased ABCA1-mediated cholesterol efflux to plasma

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Abstract

Apolipoprotein (apoE) plays an important role in lipoprotein metabolism. This study investigated the effects of adenovirus-mediated human apoE overexpression (AdhApoE3) on sterol metabolism and in vivo reverse cholesterol transport (RCT).
In wild-type mice, AdhApoE3 resulted in decreased HDL cholesterol levels and a shift toward larger HDL in plasma, whereas hepatic cholesterol content increased ($P < 0.05$). These effects were dependent on scavenger receptor class B type I (SR-BI) as confirmed using SR-BI-deficient mice. Kinetic studies demonstrated increased plasma HDL cholesteryl ester catabolic rates ($P < 0.05$) and higher hepatic selective uptake of HDL cholesteryl esters in AdhApoE3-injected wild-type mice ($P < 0.01$). However, biliary and fecal sterol output as well as in vivo macrophage-to-feces RCT studied with 3H-cholesterol-loaded mouse macrophage foam cells remained unchanged upon human apoE overexpression. Similar results were obtained using hApoE3 overexpression in human CETP transgenic mice. However, blocking ABCA1-mediated cholesterol efflux from hepatocytes in AdhApoE3-injected mice using probucol increased biliary cholesterol secretion ($P < 0.05$), fecal neutral sterol excretion ($P < 0.05$), and in vivo RCT ($P < 0.01$), specifically within neutral sterols.
These combined data demonstrate that systemic apoE overexpression increases i) SR-BI-mediated selective uptake into the liver and ii) ABCA1-mediated efflux of RCT-relevant cholesterol from hepatocytes back to the plasma compartment, thereby resulting in unchanged fecal mass sterol excretion and overall in vivo RCT.

Introduction

Apolipoprotein E (apoE) plays an important role in lipoprotein metabolism and atherosclerosis. ApoE is produced and secreted predominantly by the liver [1], but it is also expressed in a variety of other tissues, including macrophages [2, 3]. While loss of function of apoE in mice and in humans is associated with a proatherogenic lipoprotein profile and increased atherogenesis [4, 5], overexpression of apoE in various models has been shown to protect against atherosclerotic lesion formation [6-11]. Among other metabolic effects that are potentially antiatherogenic, apoE has been reported to promote cholesterol efflux [12-14], and recent studies have suggested that lack of macrophage apoE might decrease overall reverse cholesterol transport (RCT) [15]. However, the pool of macrophage-derived apoE represents a small fraction of total circulating apoE.

The classic RCT pathway is a multistep process that involves i) HDL-mediated efflux of excess cholesterol from extrahepatic cells and most relevant for atherosclerosis lipid-laden macrophages in the arterial wall, ii) uptake of HDL cholesterol into the liver, and iii) excretion of HDL cholesterol into bile and ultimately feces either directly or after metabolic conversion into bile acids [16-18]. Although the liver has a key function in the RCT pathway and the majority of circulating apoE is generated by hepatocytes, the contribution of hepatic apoE to in vivo RCT has not been addressed. Therefore, the aim of the current study was to investigate the effects of hepatic overexpression of human apoE on liver lipid metabolism, biliary sterol secretion, and in vivo macrophage-to-feces RCT. Our data demonstrate that increasing plasma levels of liver-derived apoE enhances selective uptake of HDL cholesteryl esters into the liver and induces hepatic cholesterol accumulation in a scavenger receptor class B type I (SR-BI)-dependent manner. However, this does not affect fecal mass sterol excretion and macrophage-specific RCT due to an apoE-induced enhancement of ATP-binding cassette transporter A1 (ABCA1)-mediated efflux of RCT-relevant cholesterol from hepatocytes back to the plasma compartment. These findings suggest that systemic apoE overexpression protects against atherosclerosis by mechanisms other than modulation of RCT.

Materials and Methods

Animals

C57BL/6J mice were obtained from Charles River (Wilmington, MA). SR-BI knockout mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and backcrossed to the C57BL/6J background for a total of eight generations. Probucol (Sigma, St. Louis, MO) was mixed into powdered chow (0.5% wt/wt). For the RCT experiment, the diet was provided for 12 days before and then throughout the 48-h period of the experiment. In all other experiments, the diet was provided for 14 days. Animals were caged in animal rooms with alternating 12-h periods of light (from

7:00 AM to 7:00 PM) and dark (from 7:00 PM to 7:00 AM), with ad libitum access to water and mouse chow diet (Arie Blok, Woerden, The Netherlands). Animal experiments were performed in conformity with PHS policy and in accordance with the national laws. All protocols were approved by the responsible ethics committee of the University of Groningen and the University of Pennsylvania.

Generation of recombinant adenoviruses

The empty control adenovirus AdNull [19] and the recombinant adenovirus encoding human apoE3 (AdhApoE3) [19] were amplified and purified as reported previously [20]. For in vivo experiments, mice were injected with 1 × 1011 particles/mouse of AdhApoE3 or AdNull. In vivo reverse cholesterol transport studies were carried out between day 2 and day 4 after injection of recombinant adenoviruses, a time frame when high and stable expression from an adenovirus is achieved. All other experiments described were performed on day 4 after injection of the recombinant adenoviruses.

Plasma lipid and lipoprotein analysis

Mice were bled by heart puncture after a 4-h fast at the time of death. Aliquots of plasma were stored at −80°C until analysis. Commercially available reagents were used to measure plasma total cholesterol, triglycerides (Roche Diagnostics, Basel, Switzerland), free cholesterol, and phospholipids (Diasys, Holzheim, Germany). Pooled plasma samples were subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column (GE Healthcare, Uppsala, Sweden) as described [21]. Individual fractions were assayed for cholesterol concentrations as described above.

To determine plasma HDL- and nonHDL-cholesterol levels, apoB-containing lipoproteins were precipitated using polyethylene glycol in 10 mM HEPES (pH 8.0) as described [22]. After centrifugation of the samples at 2,000 g and 4°C for 30 min, the HDL-containing supernatant was transferred to clean tubes. The nonHDL-containing pellet was dissolved in 0.5 M NaCO3. Cholesterol concentrations in the HDL and nonHDL fractions were determined as described above.

Analysis of liver lipid composition

Liver tissue was homogenized as described [23]. Commercially available kits were used to measure contents of total cholesterol, triglycerides (Roche Diagnostics), and free cholesterol (Diasys) after extraction of lipids according to the general procedure of Bligh Dyer and redissolving lipids in water containing 2% Triton X-100 [23]. Phospholipid content of the liver was determined after lipid extraction essentially as described [23].

Analysis of gene expression by real-time quantitative PCR

Total RNA from mouse livers was extracted with TriReagent (Sigma) and quantified using a Nanodrop ND-100 U-Vis spectrophotometer (NanoDrop Technologies,

Wilmington, DE). cDNA synthesis was performed from 1 μg of total RNA using reagents from Invitrogen (Carlsbad, CA). Real-time quantitative PCR was carried out on an ABI-Prism 7700 (Applied Biosystems, Darmstadt, Germany) sequence detector with the default settings [23]. Primers and fluorogenic probes were designed with the Primer Express Software (Applied Biosystems) and synthesized by Eurogentec (Seraing, Belgium). mRNA expression levels presented were calculated relative to the average of the housekeeping gene cyclophilin and further normalized to the relative expression levels of the respective controls.

Bile collection and assessment of biliary excretion of bile acids, phospholipids, and cholesterol

Bile was collected by cannulation of the gallbladder in mice anesthetized by intraperitoneal injection of hypnorm (fentanyl/fluanisone, 1 mg/kg) and diazepam (10 mg/kg). During the bile collection, body temperature was maintained using a humidified incubator. Bile collection was performed for 30 min, and secretion rates were determined gravimetrically. Biliary bile salt, cholesterol, and phospholipid concentrations were determined and the respective biliary excretion rates calculated as described previously [23, 24].

Fecal sterol analysis

Mice were individually housed, and feces were collected over a period of 24 h and separated from the bedding. Fecal samples were dried, weighed, and thoroughly ground. Aliquots thereof were used for determination of neutral sterol and bile acid content by gas-liquid chromatography as described [23, 24].

HDL kinetics studies

HDL kinetics studies were performed essentially as published previously [21]. Autologous HDL was prepared from pooled mouse plasma by sequential ultracentrifugation (density $1.063 < d < 1.21$). After extensive dialysis against sterile PBS containing 0.01% EDTA, HDL was labeled with 125I-tyramine-cellobiose (TC) and cholesteryl hexadecyl ether (cholesteryl-1,2,-3H; Perkin Elmer Life Sciences) as previously described [25]. For kinetic studies, 0.4 μCi of 125I and 0.7 million dpm of the 3H tracer were injected into the tail veins of fasted wild-type mice treated with AdNull or AdhApoE3. Blood samples were drawn by retroorbital bleeding at 5 min and at 1, 3, 6, 11, and 24 h after injection. Plasma decay curves for both tracers were generated by dividing the plasma radioactivity at each time point by the radioactivity at the initial 5-min time point after tracer injection. Fractional catabolic rates (FCRs) were determined from the area under the plasma disappearance curves fitted to a bicompartmental model using the SAAM II program [26]. The use of 3H-cholesteryl ether does not affect turnover rates in vivo compared with 3H-cholesteryl ester-labeled HDL [21]. Organ uptake of HDL apolipoproteins (125I) and HDL-CEs (3H-cholesteryl ether) was determined by measuring the counts recovered in each organ expressed as a percentage of the injected dose, which was calculated by multiplying the initial

plasma counts (5-min time point) with the estimated plasma volume (3.5% of total body weight). Selective uptake into organs was determined by subtracting the percentage of the injected dose of 125I-HDL recovered in each organ from the percentage of the injected dose of 3H-HDL-CE.

In vivo RCT studies

In vivo RCT studies were performed essentially as published previously [27]. Wild-type C57BL/6J donor mice were injected with 1.0 ml of 4% Brewer thioglycollate medium (Becton Dickinson, Le Point de Claix, France). Peritoneal macrophages were harvested 4 days after thioglycollate injection as described [28]. Macrophages were plated in RPMI 1640 medium (Invitrogen) supplemented with 1% FBS (HyClone, Logan, UT) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Invitrogen) and were allowed to adhere for 5 h at 37°C under 5% CO2 humidified air. Nonadherent cells were removed by washing twice with PBS followed by loading of the macrophages with 50 µg/ml acetylated LDL and 3 µCi/ml 3H-cholesterol (Perkin Elmer Life Sciences, Boston, MA) for 24 h. Thereafter, cells were washed again and equilibrated in RPMI 1640 medium supplemented with 2% BSA (Sigma) for 18 h. Immediately before injection, cells were harvested and resuspended in RPMI 1640 medium. For in vivo macrophage-to-feces RCT studies, 2 million 3H-cholesterol-loaded macrophage foam cells were injected intraperitoneally into individually housed recipient mice. Plasma was collected 6 h and 24 h after macrophage injection by retroorbital puncture and for the final blood draw (48 h) by heart puncture. At the end of the experimental period, livers were harvested, snap-frozen in liquid nitrogen, and stored at −80°C until further analysis. Feces were collected continuously for 48 h. Counts in plasma were assessed directly by liquid scintillation counting (Packard 1600CA Tri-Card, Packard, Meriden, CT). Counts within liver were determined after solubilization of the tissue using Solvable (Packard) exactly as previously reported [26]. Counts recovered from the respective liver piece were back-calculated to total liver mass. Feces were separated from the bedding, dried, weighed, and thoroughly ground. Aliquots were separated into neutral sterol and bile acid fractions as previously reported [27]. Briefly, samples were heated for 2 h at 80°C in alkaline methanol and then extracted three times with petroleum ether. In the top layer, radioactivity within the neutral sterol fraction was determined by liquid scintillation counting, whereas radioactivity incorporated into bile acids was assessed from the bottom layer. Counts recovered from the respective aliquots were related to the total amount of feces produced over the whole experimental period. All obtained counts were expressed relative to the administered dose.

In vitro cholesterol efflux assay

Thioglycollate-elicited peritoneal mouse macrophages were harvested as described above. Macrophages were plated in RPMI 1640 medium (Invitrogen) supplemented with 1% FBS (HyClone, Logan, UT) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Invitrogen) and were allowed to adhere for 5 h at 37°C under 5% CO2 hu-

midified air. Nonadherent cells were removed by washing twice with PBS followed by loading of the macrophages with 50 μg/ml LDL and 1 μCi/ml 3H-cholesterol (Perkin Elmer Life Sciences, Boston, MA) for 24 h. The cells were washed again and equilibrated in RPMI 1640 medium supplemented with 2% BSA (Sigma) for 18 h. The cells were washed with PBS, and 2% mouse plasma was added. After 4 h and 8 h, radioactivity within the medium was determined by liquid scintillation counting. The cell layer was washed twice with PBS, and 0.1 M NaOH was added. Plates were incubated 30 min at room temperature, and the radioactivity remaining within the cells was assessed by liquid scintillation counting. Wells incubated with RPMI without added plasma were used as blanks to determine plasma-independent efflux, and these values were subtracted from the respective experimental values. Efflux is given as the percentage of counts recovered from the medium in relation to the total counts present on the plate (sum of medium and cells).

Statistical analysis
Statistical analyses were performed using the Statistical Package for Social Sciences version 16.0 (SPSS Inc., Chicago, IL). Data are presented as means ± SEM. The Mann-Whitney U-test was used to compare different groups. Statistical significance for all comparisons was assigned at $P < 0.05$.

Results

Hepatic apoE overexpression affects HDL size distribution but not plasma lipid levels
To assess the effects of hepatic overexpression of human apoE3 on plasma lipid levels, wild-type mice were injected with an empty control adenovirus AdNull or with an adenovirus expressing human apoE3. Plasma levels of total cholesterol, free cholesterol, esterified cholesterol, phospholipids, and triglycerides remained essentially unchanged in response to hepatic apoE overexpression (Table 1). However, FPLC analysis revealed a lower HDL cholesterol peak and a shift toward larger particles in the AdhApoE3-injected mice compared with controls (Figure 1A). In parallel, plasma levels of apoA-I ($P = 0.055$; Supplementary figure IA) and apoB100 ($P < 0.01$; Supplementary figure IB) were lower in the mice overexpressing human apoE, whereas plasma apoB48 was not altered (n.s.; Supplementary figure IC). To explore the distribution of human apoE across the different lipoprotein classes, Western blot analysis for apoA-I and human apoE was performed on the individual FPLC fractions. In the mice administered AdhApoE3, human apoE was present in the apoA-I-containing HDL fractions and in the nonHDL lipoprotein fractions lacking apoA-I expression (Supplementary figure II). Because cholesteryl ester transfer protein (CETP) plays an important role in human lipoprotein metabolism but is absent in wild-type mice [29], apoE overexpression experiments were carried out in transgenic mice expressing human CETP under the control of its endogenous promoter (hCETP tg). Comparable to the results in wild-type mice, in hCETP tg mice no major changes in plasma

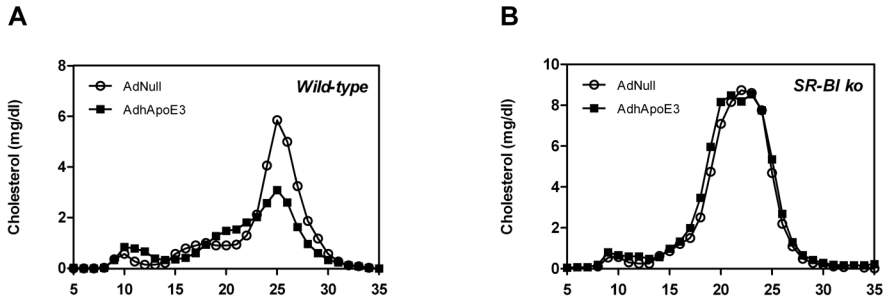

Figure 1: Apolipoprotein E overexpression affects plasma cholesterol distribution in an SR-BI-dependent fashion. FPLC profiles in response to apolipoprotein E overexpression in (A) wild-type mice and (B) SR-BI knockout (ko) mice. Pooled plasma samples collected on day 4 after injection with the control adenovirus AdNull or with the human apolipoprotein E3 expressing adenovirus AdhApoE3 were subjected to gel filtration chromatography analysis using a Superose 6 column as described in Materials and Methods. n = 6–8 mice for each condition. Open circles, AdNull-injected controls; closed squares, AdhApoE3-injected mice.

lipids occurred in response to hepatic apoE overexpression (Supplementary table I), and the HDL cholesterol peak was similarly decreased and was shifted toward larger HDL particles (Supplementary figure III).

Hepatic apoE overexpression increases hepatic cholesterol content by stimulating selective uptake into the liver

Next, we determined whether hepatic overexpression of human apoE would affect hepatic lipid composition. Hepatic total cholesterol content was significantly increased by 24% in mice administered AdhApoE3 ($P < 0.05$; Table 1), largely due to a higher hepatic esterified cholesterol content (+150%; $P < 0.01$; Table 1). Whereas hepatic phospholipids were identical between AdNull-injected and AdhApoE3-injected mice (Table 1), apoE overexpression resulted in an elevated hepatic triglyceride content (+355%; $P < 0.01$; Table 1). Similar changes in hepatic cholesterol and triglyceride content were observed in response to AdhApoE3 in hCETP tg mice (Supplementary table II).

To test the hypothesis that the decrease in plasma HDL cholesterol and the concomitant increase in hepatic cholesterol content in response to apoE overexpression were due to an enhanced selective uptake of cholesteryl esters from HDL, HDL kinetic studies were carried out in wild-type mice using autologous HDL. Hepatic apoE overexpression caused an increase in the HDL cholesteryl ester FCR (0.142 ± 0.009 vs. 0.196 ± 0.013 pools/h; $P < 0.05$; Figure 2A) without having significant effects on the HDL protein FCR (0.084 ± 0.007 vs. 0.091 ± 0.013 pools/h; n.s.; Figure 2A). Therefore, the apparent whole body selective uptake as calculated by the difference between the HDL cholesteryl ester and HDL protein FCRs was significantly higher in apoE-overexpressing mice compared with controls (0.058 ± 0.011 vs. 0.106 ± 0.010 pools/h; $P < 0.05$; Figure 2A). In agreement with the above results, the uptake of HDL protein into the liver remained unchanged after hepatic apoE overexpression

Table 1: Plasma lipids, liver lipid composition, and biliary excretion of sterols in wild-type and SR-BI knockout mice in response to hepatic apolipoprotein E overexpression.

	Wild-type		SR-BI knockout	
	AdNull	**AdhApoE3**	**AdNull**	**AdhApoE3**
Plasma				
Total cholesterol (mg/dl)	69.5 ± 1.9	65.5 ± 2.6	147.6 ± 9.5	140.5 ± 12.1
Free cholesterol (mg/dl)	29.0 ± 0.7	31.3 ± 0.9	86.4 ± 4.9	81.3 ± 7.8
Esterified cholesterol (mg/dl)	40.5 ± 1.6	34.3 ± 3.1	61.1 ± 6.4	59.2 ± 5.4
Phospholipids (mg/dl)	178.6 ± 12.7	148.6 ± 8.0	191.8 ± 10.8	214.7 ± 16.5
Triglycerides (mg/dl)	82.9 ± 9.2	80.0 ± 8.9	59.8 ± 6.9	79.9 ± 10.8
Liver				
Total cholesterol (nmol/mg liver)	6.3 ± 0.5	7.8 ± 0.4[a]	8.5 ± 0.3	8.3 ± 0.4
Free cholesterol (nmol/mg liver)	5.4 ± 0.4	5.9 ± 0.3	6.7 ± 0.1	6.4 ± 0.1
Esterified cholesterol (nmol/mg liver)	0.8 ± 0.2	2.0 ± 0.1[a]	1.7 ± 0.3	2.0 ± 0.3
Phospholipids (nmol/mg liver)	28.2 ± 1.5	25.8 ± 0.9	31.9 ± 0.7	29.5 ± 0.6[a]
Triglycerides (nmol/mg liver)	12.8 ± 3.1	58.3 ± 5.0[a]	22.2 ± 1.6	32.8 ± 4.5
Bile				
Bile flow (µl/min/100 g bw)	10.3 ± 0.6	10.2 ± 0.2	ND	ND
Biliary bile acid secretion (nmol/min/100 g bw)	866 ± 86	762 ± 45	ND	ND
Biliary phospholipid secretion (nmol/min/100 g bw)	61.6 ± 5.0	82.6 ± 6.8[a]	ND	ND
Biliary cholesterol secretion (nmol/min/100 g bw)	3.9 ± 0.2	4.0 ± 0.3	ND	ND

On day 4 following adenovirus injection bile was collected continuously for 30 minutes, plasma samples were taken, and livers were harvested and snap-frozen in liquid nitrogen. Plasma lipids, liver lipids, and biliary output rates of bile acids, phospholipids, and cholesterol were determined as described under Materials and Methods. Values are means ± SEM; n = 5-8 mice for each condition. AdhApoE3, recombinant adenovirus expressing human apoE3; AdNull, empty control adenovirus; SR-BI, scavenger receptor class B type I; bw, body weight; ND = not determined. a significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

(26.2 ± 3.7 vs. 24.6 ± 3.3%; n.s.; Figure 2B), whereas uptake of HDL cholesteryl ester into the liver tended to be higher (40.4 ± 2.4 vs. 52.8 ± 4.2%; $P = 0.07$; Figure 2B). Overall, this translated into an almost 2-fold increase in hepatic selective uptake in the AdhApoE3-injected group (14.2 ± 2.7 vs. 28.2 ± 1.6; $P < 0.01$; Figure 2B). Although selective uptake of HDL cholesteryl esters in the liver was enhanced, Sr-b1 mRNA expression was lower in wild-type mice ($P < 0.01$; Table 2) and in hCETP tg mice (Supplementary table III) overexpressing apoE. However, neither total nor membrane-associated hepatic SR-BI protein levels were changed in the two mouse models (Supplementary figure IV). Combined, these data demonstrate that hepatic apoE overexpression increases hepatic cholesterol content by stimulating selective uptake of HDL cholesteryl esters into the liver.

Increased hepatic cholesterol content in response to hepatic apoE overexpression is dependent on SR-BI

SR-BI is the major receptor responsible for the selective uptake of HDL cholesterol

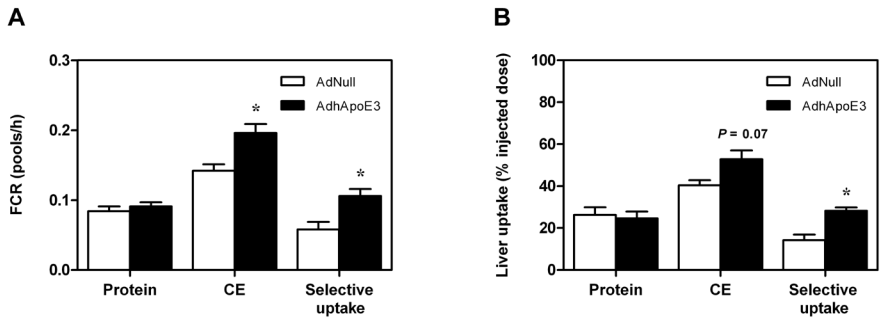

Figure 2: Apolipoprotein E overexpression increases selective uptake of HDL cholesteryl esters into the liver. On day 4 after injection with the control adenovirus AdNull or with the human apolipoprotein E3-expressing adenovirus AdhApoE3 kinetic experiments were performed using autologous HDL double labeled with 125I-tyramine-cellobiose and 3H-cholesteryl ether (CE) as described in Materials and Methods. (A) FCRs calculated from the respective plasma disappearance curves. (B) Uptake of 125I-tyramine-cellobiose and 3H-CE by the liver. Data are presented as means ± SEM. n = 6 mice for each condition. White bars, AdNull-injected mice; black bars, AdhApoE3-injected mice. * Significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

into the liver [21, 30]. To confirm a critical role of SR-BI mediating altered plasma lipoprotein distribution and hepatic cholesterol content as a consequence of apoE overexpression, the effects of AdNull or AdhApoE3 were investigated in SR-BI-deficient mice. In agreement with results in wild-type mice, injection of a human apoE expressing adenovirus did not alter plasma levels of total cholesterol, free cholesterol, esterified cholesterol, phospholipids, and triglycerides in SR-BI knockout mice (Table 1). However, the marked alterations observed in the lipoprotein distribution in response to apoE overexpression in wild-type mice were not present in SR-BI knockouts, as reflected by virtually identical FPLC profiles in the AdNull-injected compared with the AdhApoE3-injected group (Figure 1B). In line with these results, the hepatic content of total cholesterol (Table 1), free cholesterol (Table 1), and esterified cholesterol (Table 1) was not affected by apoE overexpression in SR-BI knockout mice. Nevertheless, AdhApoE3 injection in the SR-BI knockouts caused a slight but significant decrease in hepatic phospholipid content (–8%; $P < 0.05$; Table 1), whereas the hepatic triglyceride content tended to be higher (+48%; $P = 0.06$; Table 1). These data indicate that the apoE-mediated changes in lipoprotein distribution and hepatic cholesterol content are dependent on SR-BI.

Hepatic apoE overexpression does not affect biliary and fecal sterol excretion

To explore whether higher SR-BI-mediated hepatic cholesterol uptake after apoE overexpression in wild-type mice would translate into changes in biliary sterol secretion, a continuous bile cannulation experiment was performed in wild-type mice receiving AdNull or AdhApoE3. Neither bile flow (Table 1) nor biliary secretion rates of bile acids (Table 1) were affected by hepatic overexpression of human apoE3. Al-

Table 2: Hepatic mRNA expression in wild-type mice in response to hepatic apolipoprotein E overexpression.

	Wild-type	
	AdNull	**AdhApoE3**
Sr-b1	1.00 ± 0.03	0.70 ± 0.03[a]
Abcb11	1.00 ± 0.06	0.60 ± 0.04[a]
Abcb4	1.00 ± 0.07	0.91 ± 0.05
Abcg5	1.00 ± 0.08	0.62 ± 0.04[a]
Abcg8	1.00 ± 0.06	0.69 ± 0.06[a]
Cyp7a1	1.00 ± 0.23	0.91 ± 0.13
Cyp27a1	1.00 ± 0.09	0.46 ± 0.04[a]
Cyp8b1	1.00 ± 0.07	0.74 ± 0.02[a]
Srebp2	1.00 ± 0.10	0.70 ± 0.02[a]
Ldlr	1.00 ± 0.09	0.71 ± 0.04
Hmgcr	1.00 ± 0.16	0.74 ± 0.09
Abca1	1.00 ± 0.02	0.92 ± 0.06

Livers of mice administered the respective adenoviruses were harvested on day 4 after adenovirus injection and snap-frozen in liquid nitrogen. mRNA expression levels were determined by real-time quantitative PCR as described in Materials and Methods. Values are means ± SEM; n = 6 mice for each condition. Within each set of experiments, gene expression levels are related to the respective AdNull-injected controls. AdhApoE3, recombinant adenovirus expressing human apoE3; AdNull, empty control adenovirus. a significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

though the biliary secretion rate of phospholipids was 1.3-fold higher ($P < 0.05$; Table 1), the secretion rate of cholesterol into bile remained unchanged in wild-type mice (Table 1). However, in hCETP tg mice, lower biliary output of cholesterol was noted in the group injected with AdhApoE3, whereas there was no effect on the biliary secretion rates of bile acids and phospholipids (Supplementary figure V).

Hepatic mRNA expression of the hepatobiliary phospholipid transporter Abcb4 (also known as multidrug resistance protein 2, Mdr2) was not changed by apoE overexpression in wild-type mice, whereas expression levels of the bile salt export pump Abcb11 (also known as Bsep; $P < 0.01$) and the cholesterol half-transporters Abcg5 and Abcg8 were decreased ($P < 0.01$ for both) (Table 2). ApoE overexpression reduced expression of the key enzyme for the alternative bile acid synthesis pathway in the liver, Cyp27a1 ($P < 0.01$) and the enzyme responsible for cholate synthesis, Cyp8b1 ($P < 0.05$) but did not affect expression of the rate-limiting enzyme for classic bile acid synthesis, Cyp7a1 (Table 2). Hepatic gene expression of the sterol regulatory binding protein 2 (Srebp2) was suppressed by apoE overexpression ($P < 0.05$), whereas mRNA expression of its two target genes LDL receptor (Ldlr) and the rate-limiting enzyme for cholesterol synthesis, HMG-CoA reductase (Hmgcr), was not significantly affected (Table 2). There was also no difference in Abca1 mRNA levels in the liver between AdhApoE3 injected wild-type mice and controls. In hCETP tg mice, virtually identical changes in liver gene expression were found in response to

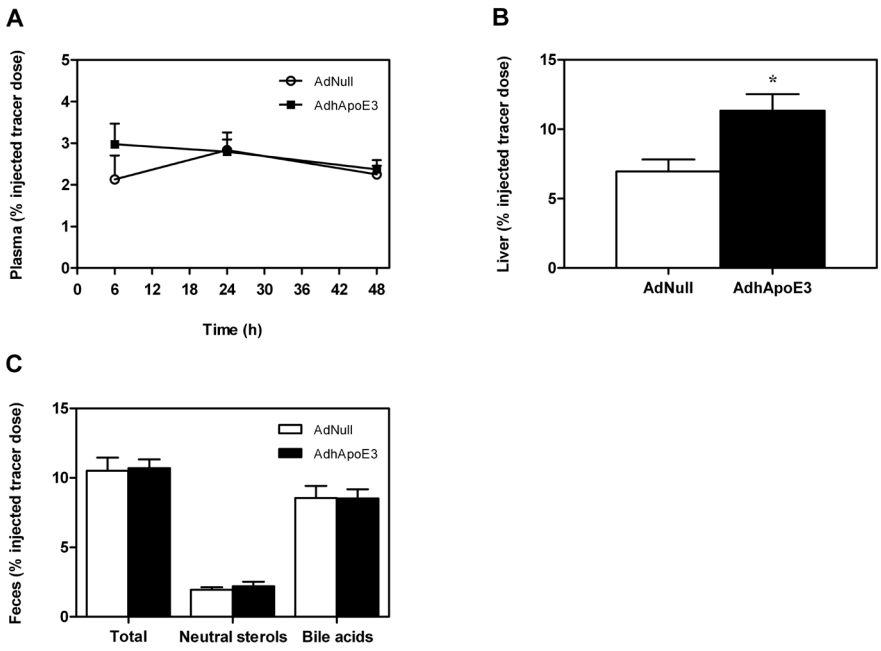

Figure 3: Apolipoprotein E overexpression does not affect in vivo macrophage-to-feces reverse cholesterol transport in wild-type mice. On day 2 after injection with the control adenovirus AdNull or with the human apolipoprotein E3-expressing adenovirus AdhApoE3 mice received intraperitoneal injections with 3H-cholesterol-loaded primary mouse macrophage foam cells as described in Materials and Methods. (A) Time course of 3H-cholesterol recovery in plasma. (B) 3H-cholesterol within liver 48 h after macrophage administration. (C) 3H-cholesterol appearance in feces collected continuously from 0 to 48 h after macrophage administration and separated into bile acid and neutral sterol fractions as indicated. Data are expressed as percentage of the injected tracer dose and presented as means ± SEM. n = 8 mice for each condition. White bars, AdNull-injected mice; black bars, AdhApoE3-injected mice. * Significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

apoE overexpression (Supplementary table III).
Consistent with unaltered biliary secretion of cholesterol and bile acids in wild-type mice, the fecal mass output of neutral sterols (3.11 ± 0.22 vs. 2.49 ± 0.34 μmol/day; Supplementary table IV) and bile acids (2.33 ± 0.19 vs. 2.65 ± 0.11 μmol/day; Supplementary table V) was not influenced by overexpression of apoE. Likewise, hCETP tg mice injected with AdhApoE3 also excreted similar amounts of neutral sterols (Supplementary table IV) and bile acids (Supplementary table V) into the feces compared with AdNull administered controls. Taken together, these results indicate that apoE overexpression promotes hepatic cholesterol uptake without increasing biliary and fecal sterol excretion.

Hepatic apoE overexpression does not affect macrophage-to-feces RCT
Because apoE overexpression increased hepatic selective uptake via SR-BI, an impor-

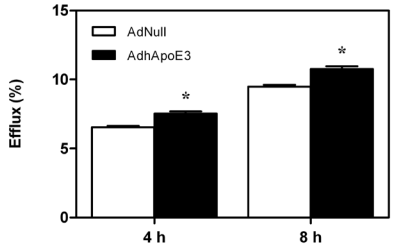

Figure 4: Apolipoprotein E overexpression increases cholesterol efflux from macrophage foam cells toward plasma. Thioglycollate-elicited peritoneal mouse macrophages were loaded with 50 µg/ml acetylated LDL and 1 µCi/ml 3H-cholesterol as described in Materials and Methods. Subsequently, 2% plasma was added to the cells. After 4 h and 8 h, radioactivity within the medium and radioactivity remaining within the cells was determined by liquid scintillation counting. Efflux is given as the percentage of counts recovered from the medium in relation to the total counts present on the plate (sum of medium and cells). Data are presented as means ± SEM. n = 8 mice for each condition. White bars, AdNull-injected mice; black bars, AdhApoE3-injected mice. * Significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

tant step in the RCT pathway, but did not influence mass biliary and fecal sterol excretion, we next investigated whether apoE overexpression might affect overall RCT. In vivo RCT was traced after intraperitoneal injection of primary mouse macrophages loaded with 3H-cholesterol in control and apoE-overexpressing wild-type mice. The appearance of tracer in plasma was not significantly different between controls and mice injected with AdhApoE3 at 6 h (2.1 ± 0.6 vs. 3.0 ± 0.5% injected tracer dose; n.s.), 24 h (2.8 ± 0.4 vs. 2.8 ± 0.3% injected tracer dose; n.s.), and 48 h (2.3 ± 0.2 vs. 2.4 ± 0.2% injected tracer dose; n.s.) after macrophage injection (Figure 3A). Although apoE overexpression led to a 63% increase in macrophage-derived 3H-cholesterol within the liver (7.0 ± 0.9 vs. 11.3 ± 1.2% injected tracer dose; $P < 0.05$; Figure 3B), overall, in vivo RCT remained essentially unchanged as reflected by no effect on the total excretion of 3H-tracer into the feces (10.5 ± 1.0 vs. 10.7 ± 0.6% injected tracer dose; n.s.; Figure 3C). In addition, no significant changes were observed in the fecal loss of 3H-tracer within neutral sterols (1.9 ± 0.2 vs. 2.2 ± 0.3% injected tracer dose; n.s.) or within the bile acid fraction (8.6 ± 0.9 vs. 8.5 ± 0.7% injected tracer dose; n.s.) in response to apoE overexpression (Figure 3C). Moreover, in the CETP transgenic mouse model, RCT was not influenced by AdhApoE3 (Supplementary figure VI). These observations indicate that hepatic apoE overexpression has no apparent effect on macrophage RCT.

Probucol treatment decreases plasma lipid and lipoprotein levels

It has been suggested recently that inhibition of hepatic ABCA1 activity by probucol reduces ABCA1-mediated cholesterol efflux from hepatocytes, thereby increasing cholesterol excretion into the bile and feces [31]. Cholesterol efflux from lipid-laden macrophages in vitro was significantly higher toward plasma from ApoE-overex-

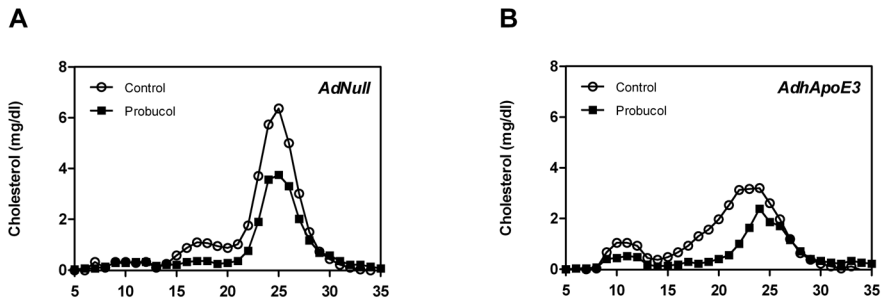

Figure 5: Probucol treatment decreases plasma cholesterol levels. FPLC profiles in response to probucol treatment in (A) AdNull-injected and (B) AdhApoE3-injected mice. Mice were fed a control chow diet or a chow diet containing 0.5% probucol for 2 weeks. Pooled plasma samples collected on day 4 after injection with the control adenovirus AdNull or with the human apolipoprotein E3-expressing adenovirus AdhApoE3 were subjected to gel filtration chromatography analysis using a Superose 6 column as described in Materials and Methods. n = 6 mice for each condition. Open circles, chow-fed controls; closed squares; probucol-treated mice.

pressing wild-type mice compared with controls after 4 h (6.5 ± 0.1 vs. 7.5 ± 0.2%; $P < 0.01$) and 8 h (9.5 ± 0.1 vs. 10.8 ± 0.2%; $P < 0.01$) of incubation (Figure 4). We therefore hypothesized that potentially increased ABCA1-mediated cholesterol efflux from hepatocytes might explain the unchanged biliary and fecal sterol output in response to apoE overexpression. To explore this hypothesis, wild-type mice were fed a control chow diet or a chow diet supplemented with 0.5% (wt/wt) probucol and were injected on day 10 of diet feeding with AdNull or AdhApoE3. Treatment with probucol decreased plasma total cholesterol concentrations in AdNull-injected (−51%; $P < 0.01$) and AdhApoE3-injected mice (−66%; $P < 0.01$; Table 3). This was due to a significant lowering of plasma free cholesterol (−52% and −71% for AdNull and AdhApoE3, respectively; $P < 0.01$ for both) as well as esterified cholesterol (−51% and −61% for AdNull and AdhApoE3, respectively; $P < 0.01$ for both) in the mice administered probucol (Table 3). As expected from the role of liver ABCA1 in HDL formation, HDL-cholesterol levels in plasma were markedly reduced by dietary probucol (−41% and −42% for AdNull and AdhApoE3, respectively; $P < 0.01$ for both). However, probucol also resulted in decreased plasma nonHDL-cholesterol levels (−66% and −79% for AdNull and AdhApoE3, respectively; $P < 0.01$ for both). In line with these results, FPLC analysis demonstrated a clear reduction in all lipoprotein classes in AdNull-injected mice fed the probucol diet compared with mice fed the control diet (Figure 5A). Similar changes in the plasma lipoprotein distribution were observed in human apoE-overexpressing mice in response to probucol (Figure 5B). Finally, administration of probucol resulted in a significant decrease in plasma phospholipids and triglycerides in mice injected with the control adenovirus AdNull (−37% and −38% for phospholipids and triglycerides, respectively; $P < 0.01$ and $P < 0.05$, respectively) or AdhApoE3 (−57% and −66% for phospholipids and triglycerides, respectively; $P < 0.01$ for both) (Table 3).

Table 3: Plasma lipids, liver lipid composition, and biliary excretion of sterols in response to probucol treatment.

	AdNull		AdhApoE3	
	Control	Probucol	Control	Probucol
Plasma				
Total cholesterol (mg/dl)	72.3 ± 1.7	35.3 ± 1.3[a]	63.7 ± 2.8	21.9 ± 1.3[a]
Free cholesterol (mg/dl)	22.2 ± 0.4	10.7 ± 0.6[a]	29.8 ± 2.0	8.5 ± 1.1[a]
Esterified cholesterol (mg/dl)	50.1 ± 1.8	24.6 ± 1.1[a]	34.0 ± 2.0	13.4 ± 0.6[a]
Phospholipids (mg/dl)	154.1 ± 7.4	97.7 ± 5.8[a]	137.8 ± 9.5	59.8 ± 1.3[a]
Triglycerides (mg/dl)	58.2 ± 7.1	36.0 ± 3.5[a]	95.3 ± 7.9	32.4 ± 7.0[a]
Liver				
Total cholesterol (nmol/mg liver)	7.2 ± 0.2	7.5 ± 0.2	9.1 ± 0.4	9.2 ± 0.5
Free cholesterol (nmol/mg liver)	5.9 ± 0.1	6.0 ± 0.2	7.4 ± 0.5	6.9 ± 0.3
Esterified cholesterol (nmol/mg liver)	1.3 ± 0.1	1.5 ± 0.1	1.7 ± 0.2	2.3 ± 0.2
Phospholipids (nmol/mg liver)	29.2 ± 0.7	26.5 ± 0.6[a]	26.7 ± 0.6	27.8 ± 0.6
Triglycerides (nmol/mg liver)	21.4 ± 1.7	22.3 ± 3.3	63.7 ± 7.1	59.4 ± 8.6
Bile				
Bile flow (µl/min/100 g bw)	9.3 ± 0.6	8.2 ± 0.7	7.3 ± 1.1	10.6 ± 0.9
Biliary bile acid secretion (nmol/min/100 g bw)	469 ± 90	357 ± 73	427 ± 81	466 ± 51
Biliary phospholipid secretion (nmol/min/100 g bw)	41.6 ± 3.1	37.7 ± 5.6	43.0 ± 5.4	64.3 ± 5.1[a]
Biliary cholesterol secretion (nmol/min/100 g bw)	3.5 ± 0.4	3.6 ± 0.5	2.9 ± 0.4	5.2 ± 0.7[a]

Mice were fed a control chow diet or a chow diet containing 0.5% probucol for 2 weeks. On day 4 after adenovirus injection, bile was collected continuously for 30 min, plasma samples were taken, and livers were harvested and snap-frozen in liquid nitrogen. Plasma lipids, liver lipids, and biliary output rates of bile acids, phospholipids, and cholesterol were determined as described in Materials and Methods. Values are means ± SEM; n = 6 mice for each condition. AdhApoE3, recombinant adenovirus expressing human apoE3; AdNull, empty control adenovirus; bw, body weight. a significantly different from the respective controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

Probucol treatment does not change the hepatic cholesterol content in response to hepatic apoE overexpression.

In mice that received the control adenovirus AdNull, the hepatic content of total cholesterol was not different between groups on control and probucol-containing diet (Table 3). ApoE overexpression consistently increased total cholesterol levels in the liver; however, there was no additional effect of probucol (Table 3). The amount of free cholesterol and esterified cholesterol in the liver was not changed in response to probucol in both the mice administered AdNull and AdhApoE3 (Table 3). Probucol treatment resulted in a lower hepatic phospholipid content in the AdNull (−9%; $P < 0.05$) but not the AdhApoE3 group (Table 3). There was no effect of probucol on the hepatic triglyceride content (Table 3). Thus, probucol does not change the hepatic cholesterol mass content in response to hepatic apoE overexpression.

Probucol treatment increases biliary and fecal sterol secretion in apoE-overexpressing mice

Bile cannulation experiments revealed that dietary probucol had no effect on bile

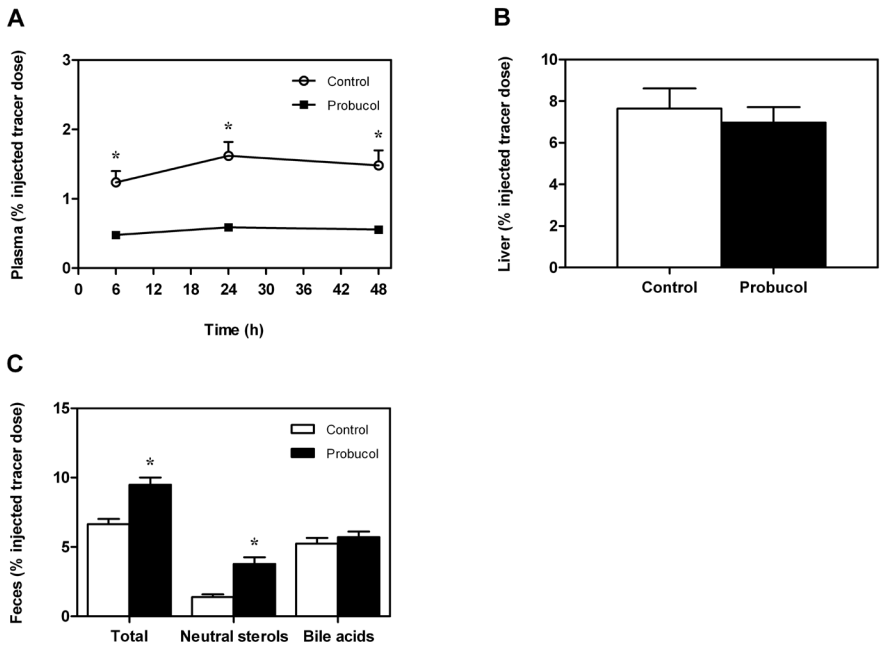

Figure 6: Probucol treatment increases in vivo macrophage-to-feces reverse cholesterol transport in apolipoprotein E-overexpressing mice. Mice were fed a control chow diet or a chow diet containing 0.5% probucol for 12 days before and then throughout the 48-h period of the experiment. On day 2 after injection with the human apolipoprotein E-expressing adenovirus AdhApoE3 mice received intraperitoneal injections with 3H-cholesterol-loaded primary mouse macrophage foam cells as described in Materials and Methods. (A) Time course of 3H-cholesterol recovery in plasma. (B) 3H-cholesterol within liver 48 h after macrophage administration. (C) 3H-cholesterol appearance in feces collected continuously from 0 to 48 h after macrophage administration and separated into bile acid and neutral sterol fractions as indicated. Data are expressed as percentage of the injected tracer dose and presented as means ± SEM. n = 8 mice for each condition. White bars, chow-fed controls; black bars, probucol-treated mice. * Significantly different from the respective controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

flow or on the biliary secretion of bile acids, phospholipids, and cholesterol in mice injected with the control adenovirus (Table 3). However, in AdhApoE3-administered mice, bile flow tended to increase in response to probucol ($P = 0.055$; Table 3). Whereas the biliary output rate of bile acids remained unchanged, biliary secretion rates of phospholipids were 1.5-fold higher in apoE-overexpressing mice in response to probucol ($P < 0.05$), and the biliary secretion rate of cholesterol increased significantly by 1.8-fold ($P < 0.05$) (Table 3).

Hepatic mRNA expression of the bile acid transporter Abcb11, the phospholipid transporter Abcb4, and the cholesterol half-transporters Abcg5 and Abcg8 was not modified by probucol treatment in control mice or in mice that overexpress apoE in the liver (Table 4). No significant changes in the hepatic gene expression level of the bile acid synthesizing enzymes Cyp7a1, Cyp27a1, and Cyp8b1 were observed, except for higher relative mRNA levels of Cyp7a1 in apoE-overexpressing mice upon

Table 4: Hepatic mRNA expression in response to probucol treatment.

	AdNull		AdhApoE3	
	Control	Probucol	Control	Probucol
Sr-b1	1.00 ± 0.05	0.88 ± 0.03[a]	1.00 ± 0.03	1.08 ± 0.03[a]
Abcb11	1.00 ± 0.06	1.16 ± 0.04	1.00 ± 0.05	1.14 ± 0.08
Abcb4	1.00 ± 0.07	1.05 ± 0.09	1.00 ± 0.06	1.12 ± 0.05
Abcg5	1.00 ± 0.10	0.91 ±0.09	1.00 ± 0.06	1.20 ± 0.09
Abcg8	1.00 ± 0.07	0.93 ± 0.08	1.00 ± 0.06	1.13 ± 0.13
Cyp7a1	1.00 ± 0.15	0.60 ± 0.09	1.00 ± 0.13	1.58 ± 0.12[a]
Cyp27a1	1.00 ± 0.05	0.90 ± 0.03	1.00 ± 0.07	1.11 ± 0.07
Cyp8b1	1.00 ± 0.08	0.96 ± 0.09	1.00 ± 0.06	1.04 ± 0.09
Srebp2	1.00 ± 0.04	0.96 ± 0.05	1.00 ± 0.05	0.81 ± 0.06
Ldlr	1.00 ± 0.08	1.14 ± 0.08	1.00 ± 0.06	0.94 ± 0.06
Hmgcr	1.00 ± 0.07	0.98 ± 0.09	1.00 ± 0.05	0.83 ± 0.09
Abca1	1.00 ± 0.04	1.07 ± 0.02	1.00 ± 0.04	1.04 ± 0.04

Mice were fed a control chow diet or a chow diet containing 0.5% probucol for 2 weeks. Livers of mice administered the respective adenoviruses were harvested on day 4 after adenovirus injection and snap-frozen in liquid nitrogen. mRNA expression levels were determined by real-time quantitative PCR as described in Materials and Methods. Values are means ± SEM; n = 6 mice for each condition. Within each set of experiments, gene expression levels are related to the respective chow-fed controls. AdhApoE3, recombinant adenovirus expressing human apoE3; AdNull, empty control adenovirus. a significantly different from the respective controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

probucol administration ($P < 0.05$; Table 4). Furthermore, expression of Srebp2 and its target genes ldlr and hmgcr in the liver was not different between mice fed a chow diet and mice fed a probucol-enriched diet (Table 4). Finally, as has been reported previously [31], no change in the hepatic mRNA expression of Abca1 was detected in the probucol-treated mice (Table 4).

Analysis of fecal contents showed that treatment with probucol did not influence the fecal excretion of neutral sterols (4.74 ± 0.28 vs. 5.07 ± 0.18 μmol/day) and bile acids (2.52 ± 0.22 vs. 3.02 ± 0.28 μmol/day) in mice that received the control adenovirus AdNull. In contrast and in good agreement with the elevated biliary cholesterol secretion, fecal excretion of neutral sterols was significantly enhanced by probucol in mice overexpressing apoE (3.69 ± 0.21 vs. 4.97 ± 0.37 μmol/day; $P < 0.05$). Nonetheless, no effect of probucol on the fecal bile acid output was found in these mice (2.20 ± 0.14 vs. 2.66 ± 0.23 μmol/day). Combined, these data demonstrate that biliary and fecal sterol secretion are increased upon probucol treatment in apoE-overexpressing mice.

Probucol treatment increases macrophage-to-feces RCT in apoE-overexpressing mice

Because probucol enhanced biliary and fecal sterol secretion in mice overexpressing human apoE, we investigated whether this would also translate into an improvement in overall RCT from macrophages to feces. After intraperitoneal injection of 3H-

cholesterol-loaded macrophages, counts within plasma were profoundly lower at the 6 h (1.24 ± 0.16 vs. 0.48 ± 0.04% injected tracer dose; P < 0.01; Figure 6A), 24 h (1.62 ± 0.20 vs. 0.59 ± 0.04% injected tracer dose; P < 0.01; Figure 6A), and 48 h time point (1.48 ± 0.22 vs. 0.56 ± 0.06% injected tracer dose; P < 0.01; Figure 6A) in the probucol-treated apoE-overexpressing mice compared with apoE-overexpressing controls. However, the amount of macrophage-derived tracer recovered within the liver was not affected by probucol in mice with hepatic apoE overexpression (7.6 ± 1.0 vs. 7.0 ± 0.7% injected tracer dose; n.s.; Figure 6B). Consistent with the higher biliary and fecal mass excretion of sterols, probucol significantly enhanced the total excretion of 3H-cholesterol originating from macrophages into the feces of AdhApoE3-injected mice (6.6 ± 0.4 vs. 9.5 ± 0.5% injected tracer dose; P < 0.01; Figure 6C). Because tracer recovery in the fecal bile acid fraction remained unaltered (5.3 ± 0.4 vs. 5.7 ± 0.4% injected tracer dose; n.s.; Figure 6C), this was attributable to a 2.7-fold higher excretion of 3H-cholesterol in the fecal neutral sterol fraction (1.4 ± 0.2% vs. 3.8 ± 0.5% injected tracer dose; P < 0.01; Figure 6C). These findings demonstrate that probucol results in an increased movement of cholesterol from macrophages to the feces in mice with hepatic apoE overexpression.

Discussion

This study demonstrates that hepatic overexpression of human apoE not only promotes SR-BI-mediated selective uptake of HDL cholesterol into the liver but also enhances the resecretion of RCT-relevant cholesterol via hepatocyte ABCA1 back to the plasma compartment. As a result, biliary and fecal mass sterol excretion and RCT remain unchanged in apoE-overexpressing mice with active ABCA1, whereas all of these parameters increase significantly when ABCA1 activity is blocked with probucol. Collectively, these results point to a metabolic shunt potentially connecting the SR-BI and the ABCA1 pathway with a high relevance for the regulation of RCT. The RCT pathway represents an important atheroprotective functionality of HDL [16, 17]. RCT comprises cholesterol efflux from macrophage foam cells within the vessel wall, the transport of this cholesterol within HDL through the plasma compartment, the subsequent hepatic uptake via SR-BI or as a holoparticle, and excretion into the bile and feces [16, 17]. In vitro, apoE expression by macrophages has been shown to stimulate cholesterol efflux [12-14]. In addition, in vivo macrophage-to-feces RCT was lower when apoE-deficient macrophages were injected into wild-type mice compared with wild-type macrophages injected into wild-type mice [15]. Combined with our present results, these data suggest that apoE expression by macrophages might determine the general availability of macrophage-derived cholesterol for the RCT pathway directly at the point of entry, whereas in subsequent steps of RCT hepatic ABCA1 counteracts these effects.

Experiments in SR-BI knockout mice as well as HDL kinetic studies in the current report demonstrated that apoE overexpression specifically increased selective up-

take of HDL cholesterol into the liver. Importantly, the expression levels of hepatic SR-BI did not change in response to apoE overexpression. Therefore, our present findings are complementary to previous work demonstrating impaired hepatic selective HDL cholesterol uptake in mice lacking apoE [32], although the apoE knockout mouse model exhibits a considerably altered plasma lipid profile with substantially increased levels of apoB-containing lipoprotein remnants.[32] In addition, selective uptake of HDL cholesterol into HepG2 cells after treatment with a blocking antibody directed against apoE was reduced [33]. It is possible that apoE stimulates SR-BI-mediated selective uptake due to an improved interaction of the HDL particle with SR-BI,[32] although the exact mechanisms will require further investigation.

Although hepatic human apoE overexpression increased the hepatic cholesterol content, biliary cholesterol excretion and the overall elimination of cholesterol from the body did not increase in wild-type mice or hCETP tg mice. The current observation that increased SR-BI-mediated selective uptake does not necessarily translate into enhanced biliary and fecal sterol excretion in the face of unaltered hepatic SR-BI expression is in agreement with earlier observations made by us in mice overexpressing group IIA secretory phospholipase A2 [23] or endothelial lipase (EL) [21, 34]. Transgenic overexpression of secretory phospholipase A2 or adenovirus-mediated overexpression of EL also resulted in an increased flux of cholesterol into the liver, whereas biliary cholesterol secretion and mass fecal excretion of sterols remained unaffected [21, 23, 34]. On the other hand, altering the hepatic expression level of SR-BI has clear effects on biliary cholesterol secretion as well as RCT with overexpression resulting in an increase and knockdown in a decrease of both of these parameters [24, 34, 35]. How can this discrepancy be explained? Recent data generated in probucol-fed mice demonstrated that a decrease in hepatic ABCA1 activity results in increased in vivo RCT [31]. We therefore speculate that increased cholesterol uptake into the liver via SR-BI results in transport to a specific intrahepatic compartment also accessible for ABCA1-mediated resecretion back into the plasma compartment to generate new HDL particles. Increasing hepatic SR-BI expression levels [24, 34] or decreasing the activity of ABCA1 might shift the balance toward biliary secretion (present report and Reference 31). However, the nature of these intrahepatic cholesterol pools is unknown. Future in vitro studies should therefore focus on the intracellular trafficking of cholesterol and, using a pulse-chase set-up, on the incorporation of HDL-derived cholesterol tracers into newly formed HDL in models with altered expression of hepatocyte ABCA1. Ideally, such studies should be carried out in polarized liver cells. To formally relate the cholesterol mass changes observed in our current study to each other, quantifying cholesterol fluxes would be required, which has not been done in our present study and thus represents a potential limitation in the interpretation of our current work.

Systemic apoE [7-11] and macrophage-derived apoE [36-39] have been shown to protect against atherosclerotic lesion formation due to effects not immediately involving RCT. ApoE promotes the clearance of atherogenic lipoproteins [6, 8, 10], and also a number of pleiotropic effects of apoE might be important. For example,

apoE has antioxidative properties. ApoE prevented oxidative cell death in cultured neuronal cells and inhibited copper-mediated oxidation of LDL, a key event in the initiation and progression of atherosclerotic lesions [40]. Moreover, apoE knockout mice have significantly increased in vivo oxidative stress, as determined by plasma, urinary, and vascular isoprostane levels [41], whereas hepatic overexpression of apoE in LDL receptor knockout mice resulted in a reduced oxidative stress burden and decreased atherosclerosis independent of plasma lipid and lipoprotein levels [9]. Anti-inflammatory activities of apoE also add to its atheroprotective properties. In vitro studies have shown that apoE can suppress proliferation of cultured peripheral blood T lymphocytes [42] and shifts polarization of mouse macrophages to the anti-inflammatory M2 phenotype [43]. In vivo, apoE-deficient mice exhibit an exaggerated proinflammatory cytokine response after LPS injection compared with wild-type mice [44, 45], which could be partially normalized by hepatic overexpression of human apoE3 [44]. In addition, apoE can prevent LPS-induced mortality in wild-type mice [45]. More recently, hepatic expression of human apoE has been shown to limit monocyte entry into the vessel wall and thereby to contribute significantly to regression of preexisting plaques in apoE knockout mice [46]. Finally, apoE might be antiatherogenic by inhibiting platelet aggregation [47] as well as proliferation of vascular smooth muscle cells [48].

In summary, this study demonstrates that hepatic overexpression of human apoE3 not only facilitates SR-BI-mediated selective uptake of HDL cholesterol into the liver but also increases ABCA1-mediated resecretion of RCT-relevant cholesterol back into the plasma compartment. Decreasing hepatocyte ABCA1 activity might therefore represent a strategy to enhance the anti-atherosclerotic efficacy of apoE. However, before applying such strategies, the underlying mechanisms need to be delineated in more detail.

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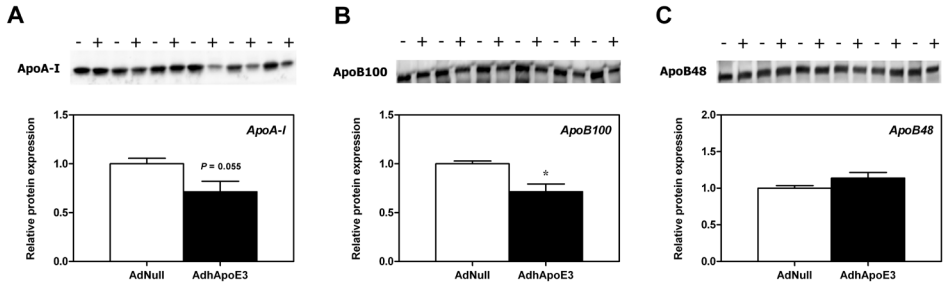
Supplemental material chapter 3

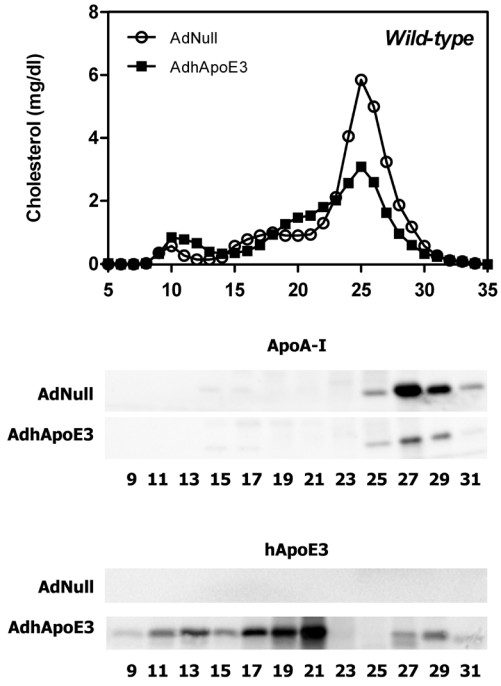
Supplemental materials and methods

Animals
Human cholesteryl ester transfer protein transgenic (hCETP tg) mice, expressing a human CETP minigene under the control of its natural flanking regions [1], originated from Dr. Alan Tall´s laboratory at Columbia University (New York, NY) and were bred at the Leiden University Medical Center, The Netherlands.

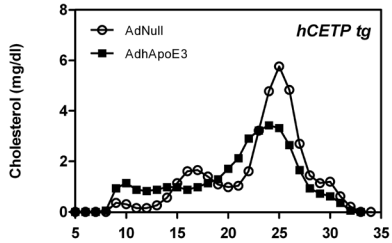
Western blotting
Western blots for apolipoprotein (apo) A-I and apoB48/100 were carried out on plasma. Western blots for apoA-I and human apoE were carried out on fast protein liquid chromatography (FPLC) fractions. Equal amounts of plasma and individual FPLC fractions were resolved by SDS-PAGE electrophoresis and blotted onto nitrocellulose. ApoA-I was visualized using a commercially available rabbit anti-mouse apoA-I antibody (Calbiochem, San Diego, CA), apoB48/100 was detected using a commercially available rabbit anti-mouse apoB48/100 antibody (Biodesign, Memphis, TN) and human apoE was visualized using a mouse monoclonal anti-human apoE3 antibody [2] each followed by the appropriate HRP-conjugated secondary antibody. HRP was detected using chemiluminescence (ECL, GE Healthcare, Piscataway, NJ). For plasma apoA-I and apoB48/100 Western blots densitometry analysis of the bands was performed using ImageJ software (National Institutes of Health, Bethesda, MD). Results were normalized to the relative expression levels of the respective controls, i.e mice receiving the control adenovirus AdNull.
Western blots for SR-BI were carried out on total liver homogenates as well as on hepatic membrane fractions prepared essentially as described [3]. Protein concentrations were determined with the bicinchoninic acid (BCA) assay (Pierce Biotechnology, Inc., Rockford, IL). Equal amounts of protein were resolved by SDS-PAGE electrophoresis and blotted onto nitrocellulose. SR-BI was visualized using a commercially available goat anti-mouse SR-BI antibody (Novus Biologicals, Littleton, CO), followed by the appropriate HRP-conjugated secondary antibody. Detection of HRP and densitometry analysis of the bands was performed as detailed above.



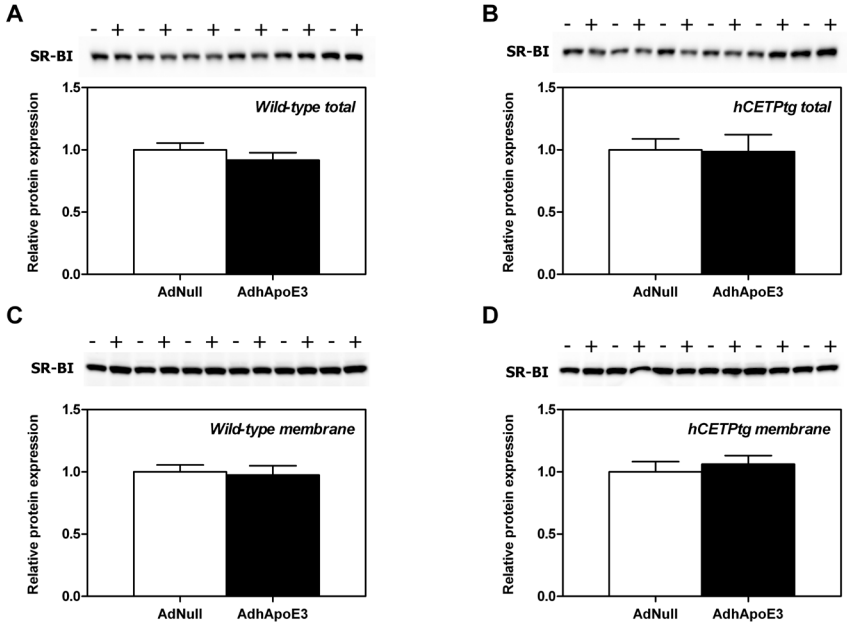
Supplemental figure I: Apolipoprotein E overexpression decreases plasma protein levels of apoA-I and apoB100. On day 4 following injection with either the control adenovirus AdNull or the human apolipoprotein E3 expressing adenovirus AdhApoE3 plasma samples were taken. Protein expression of (A) apoA-I, (B) apoB100, and (C) apoB48 in plasma was determined by Western blotting as described under Supplementary materials and methods. The upper panel shows the Western blot and the lower panel its respective quantification. Data are presented as means ± SEM. n = 6 mice for each condition. -, AdNull-injected mice; +, AdhApoE3-injected mice. White bars, AdNull-injected mice; black bars, AdhApoE3-injected mice. * Significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).



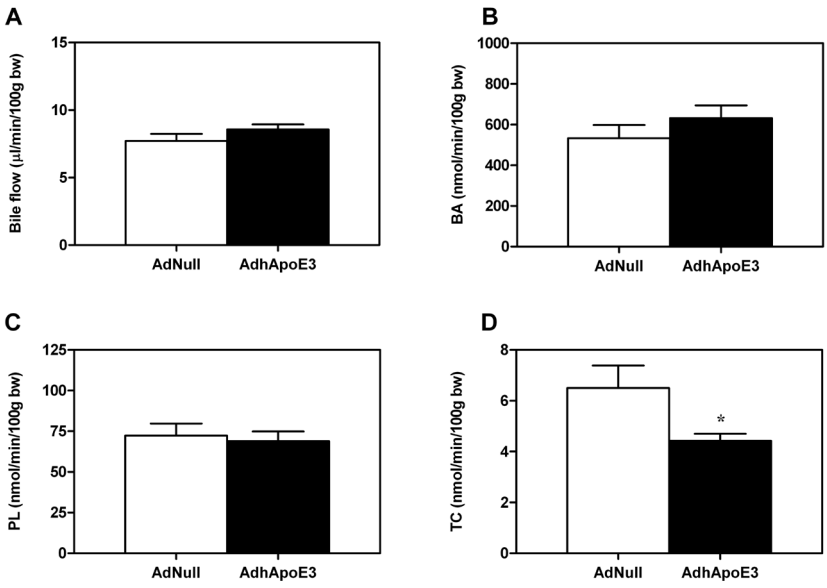
Supplemental figure II: Distribution of human apolipoprotein E over the different lipoprotein fractions. Pooled plasma samples (n = 6 mice per group) collected on day 4 following injection of wild-type mice with either the control adenovirus AdNull or the human apolipoprotein E3 expressing adenovirus AdhApoE3 were subjected to fast protein liquid chromatography (FPLC) analysis using a Superose 6 column, then cholesterol concentrations were determined. Protein expression of apoA-I and human apoE in individual fractions was determined by Western blotting as described under Supplementary materials and methods. Open circles, AdNull-injected controls; filled squares; AdhApoE3-injected mice.



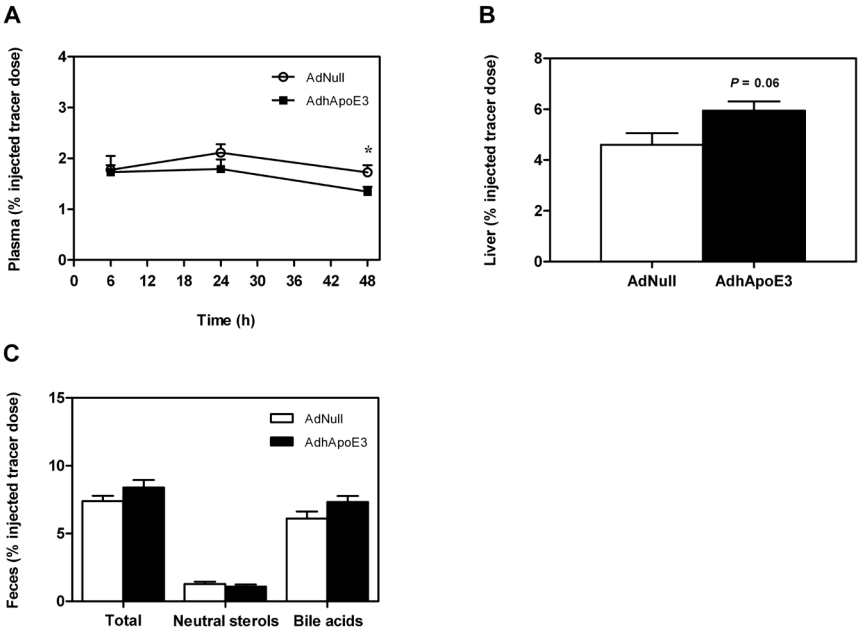
Supplemental figure III: Apolipoprotein E overexpression affects plasma cholesterol distribution over the different lipoprotein subclasses in human CETP transgenic mice. Fast protein liquid chromatography (FPLC) profiles in response to apolipoprotein E overexpression in human CETP transgenic mice (hCETP tg). Pooled plasma samples collected on day 4 following injection with either the control adenovirus AdNull or the human apolipoprotein E3 expressing adenovirus AdhApoE3 were subjected to gel filtration chromatography analysis using a Superose 6 column. n = 6 mice for each condition. Open circles, AdNull-injected controls; filled squares; AdhApoE3-injected mice.



Supplemental figure IV: Apolipoprotein E overexpression does not affect hepatic protein expression of SR-BI. On day 4 following injection with either the control adenovirus AdNull or the human apolipoprotein E3 expressing adenovirus AdhApoE3 livers were snap-frozen in liquid nitrogen. Hepatic protein expression for SR-BI was determined in total liver homogenates in (A) wild-type and (B) human CETP transgenic mice as well as in hepatic membrane fractions in (C) wild-type and (D) human CETP transgenic mice by Western blotting as described under Supplementary materials and methods. The upper panel shows the Western blot and the lower panel its quantification. Data are presented as means ± SEM. n = 6 mice for each condition. -, AdNull-injected mice; +, AdhApoE3-injected mice. White bars, AdNull-injected mice; black bars, AdhApoE3-injected mice. hCETP tg, human cholesteryl ester transfer protein transgenic mice.



Supplemental figure V: Apolipoprotein E overexpression decreases biliary cholesterol excretion in human CETP transgenic mice. Bile flow (A) and biliary secretion rates of (B) bile acids (BA), (C) phospholipids (PL), and (D) cholesterol (TC) in response to hepatic apolipoprotein E overexpression in human CETP transgenic mice. On day 4 following injection with either the control adenovirus AdNull or the human apolipoprotein E3 expressing adenovirus AdhApoE3 bile was collected continuously for 30 minutes. Biliary output rates of bile acids, phospholipids, and cholesterol were determined as described under Materials and Methods. Data are presented as means ± SEM. n = 6 mice for each condition. White bars, AdNull-injected mice; black bars, AdhApoE3-injected mice. * Significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).



Supplemental figure VI: Apolipoprotein E overexpression does not affect in vivo macrophage-to-feces reverse cholesterol transport in human CETP transgenic mice. On day 2 following injection with either the control adenovirus AdNull or the human apolipoprotein E3 expressing adenovirus AdhApoE3 mice received intraperitoneal injections with 3H-cholesterol-loaded primary mouse macrophage foam cells as described under Materials and Methods. (A) Time course of 3H-cholesterol recovery in plasma. (B) 3H-cholesterol within liver 48 h after macrophage administration. (C) 3H-cholesterol appearance in feces collected continuously from 0 to 48 h after macrophage administration and separated into bile acid and neutral sterol fractions as indicated. Data are expressed as percentage of the injected tracer dose and presented as means ± SEM. n = 8 mice for each condition. White bars, AdNull-injected mice; black bars, AdhApoE3- injected mice. * Significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

Supplemental table I: Plasma lipids in human CETP transgenic mice in response to hepatic apolipoprotein E overexpression.

	hCETP tg	
	AdNull	**AdhApoE3**
Total cholesterol (mg/dl)	54.5 ± 4.1	46.9 ± 3.5
Free cholesterol (mg/dl)	25.3 ± 3.9	28.0 ± 2.5
Esterified cholesterol (mg/dl)	29.2 ± 1.6	19.0 ± 1.4[a]
Phospholipids (mg/dl)	87.9 ± 10.1	87.1 ± 8.2
Triglycerides (mg/dl)	29.8 ± 5.3	38.7 ± 9.0

Samples were taken on day 4 following adenovirus injection, and plasma lipids were determined as described under Materials and Methods. Values are means ± SEM; n = 6 mice for each condition. AdhApoE3, recombinant adenovirus expressing human apoE3; AdNull, empty control adenovirus; hCETP tg, human cholesteryl ester transfer protein transgenic mice. a significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

Supplemental table II: Liver lipid composition in human CETP transgenic mice in response to hepatic apolipoprotein E overexpression.

	hCETP tg	
	AdNull	**AdhApoE3**
Total cholesterol (nmol/mg liver)	7.7 ± 0.1	10.4 ± 0.6[a]
Free cholesterol (nmol/mg liver)	5.6 ± 0.1	8.0 ± 0.8[a]
Esterified cholesterol (nmol/mg liver)	2.0 ± 0.1	2.5 ± 0.4
Phospholipids (nmol/mg liver)	25.5 ± 1.3	26.4 ± 0.5
Triglycerides (nmol/mg liver)	38.8 ± 2.4	78.5 ± 9.2[a]

Livers of mice administered the respective adenoviruses were harvested on day 4 following adenovirus injection and snap-frozen in liquid nitrogen. Liver lipids were measured as described under Materials and Methods. Values are means ± SEM; n = 6 mice for each condition. AdhApoE3, recombinant adenovirus expressing human apoE3; AdNull, empty control adenovirus; hCETP tg, human cholesteryl ester transfer protein transgenic mice. a significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

Supplemental table III: Hepatic mRNA expression in human CETP transgenic mice in response to hepatic apolipoprotein E overexpression.

	hCETP tg	
	AdNull	**AdhApoE3**
Sr-b1	1.00 ± 0.07	0.65 ± 0.06[a]
Abcb11	1.00 ± 0.06	0.60 ± 0.07[a]
Abcb4	1.00 ± 0.10	0.85 ± 0.03
Abcg5	1.00 ± 0.08	0.48 ± 0.06[a]
Abcg8	1.00 ± 0.07	0.62 ± 0.08[a]
Cyp7a1	1.00 ± 0.19	0.38 ± 0.07
Cyp27a1	1.00 ± 0.11	0.41 ± 0.03[a]
Cyp8b1	1.00 ± 0.20	0.48 ± 0.06[a]
Srebp2	1.00 ± 0.05	0.77 ± 0.04[a]
Ldlr	1.00 ± 0.04	0.77 ± 0.06[a]
Hmgcr	1.00 ± 0.10	0.74 ± 0.06
Abca1	1.00 ± 0.05	0.88 ± 0.07

Livers of mice administered the respective adenoviruses were harvested on day 4 following adenovirus injection and snap-frozen in liquid nitrogen. mRNA expression levels were determined by real-time quantitative PCR as described under Materials and Methods. Values are means ± SEM; n = 6 mice for each condition. Within each set of experiments, gene expression levels are related to the respective AdNull-injected controls. AdhApoE3, recombinant adenovirus expressing human apoE3; AdNull, empty control adenovirus; hCETP tg, human cholesteryl ester transfer protein transgenic mice. a significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

Supplemental table IV: Fecal excretion of neutral sterols in wild-type and human CETP transgenic mice in response to hepatic apoE overexpression.

	Wild-type		hCETP tg	
	AdNull	AdhApoE3	AdNull	AdhApoE3
Coprostanol (µmol/day)	0.55 ± 0.15	0.36 ± 0.09	0.78 ± 0.08	0.83 ± 0.09
Cholesterol (µmol/day)	2.30 ± 0.09	1.94 ± 0.27	2.77 ± 0.27	2.62 ± 0.30
Dihydrocholesterol (µmol/day)	0.25 ± 0.03	0.19 ± 0.02[a]	0.26 ± 0.01	0.23 ± 0.01
Total neutral sterols (µmol/day)	3.11 ± 0.22	2.49 ± 0.34	3.80 ± 0.34	3.67 ± 0.40

Mice of the indicated genotypes administered the respective adenoviruses were individually housed and feces were collected over a period of 24 h starting on day 3 after adenovirus injection. Fecal samples were separated from the bedding, dried, weighed, and ground. Aliquots were used to determine the content of different neutral sterol species by gas liquid chromatography as described under Materials and Methods. Values are means ± SEM; n = 6 mice for each condition. AdhApoE3, recombinant adenovirus expressing human apoE3; AdNull, empty control adenovirus; hCETP tg, human cholesteryl ester transfer protein transgenic mice. a significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

Supplemental table V: Fecal excretion of bile acids in wild-type and human CETP transgenic mice in response to hepatic apoE overexpression.

	Wild-type		hCETP tg	
	AdNull	AdhApoE3	AdNull	AdhApoE3
Allocholic acid (µmol/day)	0.067 ± 0.008	0.088 ± 0.006	ND	ND
α-Muricholic acid (µmol/day)	0.124 ± 0.016	0.124 ± 0.008	0.332 ± 0.046	0.246 ± 0.032
Deoxycholic acid (µmol/day)	0.717 ± 0.056	0.923 ± 0.060[a]	1.204 ± 0.163	1.198 ± 0.205
Cholic acid (µmol/day)	0.175 ± 0.037	0.286 ± 0.043	0.195 ± 0.041	0.247 ± 0.037
Chenodeoxycholic acid (µmol/day)	0.050 ± 0.006	0.051 ± 0.008	ND	ND
Hyodeoxycholic acid (µmol/day)	0.043 ± 0.003	0.057 ± 0.007	0.160 ± 0.018	0.135 ± 0.026
β-Muricholic acid (µmol/day)	0.240 ± 0.028	0.124 ± 0.009[a]	0.479 ± 0.070	0.568 ± 0.064
ω-Muricholic acid (µmol/day)	0.910 ± 0.069	1.021 ± 0.056	2.054 ± 0.204	1.864 ± 0.276
Total bile acids (µmol/day)	2.33 ± 0.19	2.65 ± 0.11	4.42 ± 0.48	4.26 ± 0.58

Mice of the indicated genotypes administered the respective adenoviruses were individually housed and feces were collected over a period of 24 h starting on day 3 after adenovirus injection. Fecal samples were separated from the bedding, dried, weighed, and ground. Aliquots were used to determine the content of different bile acid species by gas liquid chromatography as described under Materials and Methods. Values are means ± SEM; n = 6 mice for each condition. AdhApoE3, recombinant adenovirus expressing human apoE3; AdNull, empty control adenovirus; hCETP tg, human cholesteryl ester transfer protein transgenic mice; ND = not detectable. a significantly different from the respective AdNull-injected controls as assessed by Mann-Whitney U-test (at least $P < 0.05$).

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Chapter 4

Differential Impact of Hepatic Deficiency and Total Body Inhibition of Microsomal Triglyceride Transfer Protein on Cholesterol Metabolism and Reverse Cholesterol Transport in Mice

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Abstract

Since apolipoprotein (apo) B-containing lipoproteins are pro-atherogenic and their secretion by liver and intestine largely depends on microsomal triglyceride transfer protein (MTP) activity, MTP inhibition strategies are actively pursued. How decreasing the secretion of apoB-containing lipoproteins affects intracellular re-routing of cholesterol is unclear. Therefore, the aim of the present study was to determine the effects of reducing either systemic or liver-specific MTP activity on cholesterol metabolism and reverse cholesterol transport (RCT) using a pharmacological MTP inhibitor or a genetic model, respectively.

Plasma total cholesterol and triglyceride levels were decreased in both MTP inhibitor-treated and liver-specific MTP knockout (L-Mttp-/-) mice (each $p<0.001$). With both inhibition approaches hepatic cholesterol as well as triglyceride content was consistently increased (each $p<0.001$), while biliary cholesterol and bile acid secretion remained unchanged. A small but significant decrease in fecal bile acid excretion was observed in inhibitor-treated mice ($p<0.05$) whereas fecal neutral sterol excretion was substantially increased by 75% ($p<0.001$) conceivably due to decreased intestinal absorption. In contrast, in L-Mttp-/- mice both fecal neutral sterol and bile acid excretion remained unchanged. However, while total RCT increased in inhibitor-treated mice ($p<0.01$), it surprisingly decreased in L-Mttp-/- mice ($p<0.05$).

These data demonstrate that (i) pharmacological MTP inhibition increases RCT, an effect that might provide additional clinical benefit of MTP inhibitors, and (ii) decreasing hepatic MTP decreases RCT pointing towards a potential contribution of hepatocyte-derived VLDL to RCT.

Introduction

Cholesterol within apolipoprotein (apo) B-containing lipoproteins is a major risk factor for the development of atherosclerotic cardiovascular disease (CVD) [1, 2]. Plasma levels of apoB-containing lipoproteins are determined by hepatic production in the form of very low density lipoproteins (VLDL) and intestinal production of chylomicrons, in which cholesterol absorbed from the diet is packaged [3-5]. For the assembly of both forms of apoB-containing lipoproteins microsomal triglyceride transfer protein (MTP) expression is essential [4, 5]. This is exemplified by reduced secretion of apoB-containing lipoproteins and the subsequent accumulation of hepatic triglycerides when MTP is absent in rodent models [6-9] or in human abetalipoproteinemia, a genetic disease characterized by MTP deficiency [10]. On the other hand, overexpression of MTP increases secretion of apoB-containing lipoproteins [11, 12]. Deletion of MTP specifically in the intestine was shown to reduce plasma cholesterol levels by about 45-50% [13, 14] illustrating the contribution of the intestine to systemic lipoprotein metabolism. The key role of MTP in determining circulating levels of apoB-containing lipoproteins and their importance for atherosclerotic CVD resulted in the development of systemically active pharmacological inhibitors that have recently entered phase 3 clinical trials [15, 16]. Although a variable increase in hepatic triglyceride content was noticed, MTP inhibitors seem to have a reasonable safety profile and thus represent a viable therapeutic alternative to substantially decrease plasma levels of apoB-containing lipoproteins in patients not sufficiently responding to statin therapy [15]. In addition, antisense oligonucleotides (ASOs) have been developed that specifically target hepatic MTP expression and thereby lower, at least in mouse models, circulating levels of pro-atherogenic apoB-containing lipoproteins [17].
High density lipoproteins (HDL) on the other hand protect against atherosclerotic CVD [18]. An established function of HDL is to mediate reverse cholesterol transport (RCT), a pathway in which cholesterol from atherosclerotic lesions is transported back to the liver [19, 20]. HDL cholesterol taken up into the liver can then follow different metabolic routes. In hepatocytes it can either (i) be used for conversion into bile acids [21] or (ii) be secreted directly into the bile [21] or (iii) be resecreted within newly formed HDL particles via the action of the ATP-binding cassette transporter A1 (ABCA1) [22, 23] or (iv) be re-secreted as part of VLDL [24]. The final step of RCT is the excretion of sterols, either bile acids or cholesterol, from the body via the feces, and several studies indicated that inhibiting intestinal sterol uptake increases RCT [25-27]. How pharmacological MTP inhibition or a decrease in hepatic MTP expression, clinically relevant interventions currently persued, affect the rerouting of cholesterol between different compartments and pathways has not been investigated. Therefore, the aim of the present study was to determine the effects of decreasing either systemic or hepatocyte-specific MTP activity on cholesterol metabolism and RCT. Our data demonstrate that pharmacological MTP inhibition is beneficial by increasing RCT, whereas liver-specific MTP genetic ablation decreases RCT. These

data suggest that the metabolic effects of systemic MTP inhibition could be mainly mediated by impacting intestinal cholesterol handling. In a clinical perspective our results indicate that specifically MTP inhibition strategies that target or at least include the intestinal compartment might be favorable.

Materials and Methods

Experimental animals and treatments
C57BL/6J wild-type mice were obtained from Charles River (L'Arbresle, France). These were treated with the MTP inhibitor BMS-212122 (kindly provided by Dr. David Gordon, Bristol-Myers Squibb) at a daily oral dose of 1 mg/kg/day for 2 weeks as described, resulting in a 63% decrease in hepatic MTP activity and a 86% lower intestinal MTP activity, while hepatic and intestinal MTP protein expression remained unchanged (supplemental figure I). As a consequence hepatic VLDL as well as intestinal chylomicron production were virtually abolished (supplemental figure II). Liver-specific MTP knockout mice (L-Mttp-/-) were obtained by crossing floxed MTP knockout mice [6] with transgenic mice expressing Cre recombinase under the control of the hepatocyte-specific albumin promoter (Jackson Laboratory, Bar Harbor, ME, USA; all on the C57BL/6J background) [28]. Littermate controls not expressing cre were used for all experiments. In L-Mttp-/- no hepatic MTP protein expression was detectable and liver MTP activity was substantially lower, while intestinal MTP protein expression as well as activity remained unaltered (supplemental figure I). All mice were housed in animal rooms with alternating 12-hour periods of light (from 7:00 a.m. to 7:00 p.m.) and dark (from 7:00 p.m. to 7:00 a.m.) with ad libitum access to water and mouse chow diet (Arie Blok, Woerden, The Netherlands). Animal experiments were performed in accordance with national laws. All protocols were approved by the responsible ethics committee.

Analysis of plasma lipids and lipoproteins
Mice were fasted for 4 hours and blood was obtained by cardiac puncture under anesthesia. Aliquots of plasma were stored at -80°C until analysis. Commercially available reagents were used to measure plasma total cholesterol, triglycerides (Roche Diagnostics, Mannheim, Germany) and free cholesterol (Diasys, Holzheim, Germany). Pooled plasma samples were subjected to fast protein liquid chromatography (FPLC) gel filtration using a superose 6 column (GE Healthcare, Little Chalfont, UK) as described previously [29]. Individual fractions were assayed for cholesterol and triglyceride concentrations as detailed above.

Analysis of liver lipid composition
Liver tissue was homogenized, lipids were extracted according to the general procedure of Bligh and Dyer and redissolved in water containing 2% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) [22]. Total cholesterol, free cholesterol and triglycer-

ides were assayed as described above.

Bile collection and composition analysis
The galbladder was cannulated under hypnorm (fentanyl/fluanisone; 1 ml/kg bodyweight) and diazepam (10 mg/kg bodyweight) anesthesia, bile was collected for 30 minutes and production was determined gravimetrically. A humidified incubator was used to maintain body temperature. Biliary cholesterol, phospholipid and bile acid concentrations were determined and the respective biliary secretion rates were calculated as described [30].

Analysis of fecal neutral sterol and bile acid secretion
Feces of animals individually housed in metabolic cages were collected over a 24-hour period. Fecal samples were dried, weighed and ground. Neutral sterol and bile acid composition was determined in a 50 mg fecal aliquot by gas liquid chromatography using a published protocol [22].

Cholesterol absorption studies
Fractional cholesterol absorption was determined as described before with an adapted plasma dual isotope ratio method using blood spots obtained at 0 and 72 hours after intravenous (D7) and oral (D5) administration of stable isotopically labeled cholesterol [31].

Analysis of hepatic gene expression by real-time PCR
Total RNA from mouse livers and small intestine was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantified using a NanoDrop 2000c UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA) as described before [29]. cDNA synthesis from 1 μg of total RNA was performed using reagents from Invitrogen (Carlsbad, CA, USA). Real-time quantitative PCR was performed on a 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). PCR primers and fluorogenic probes were designed with Primer Express Software (Applied Biosystems, Foster City, CA, USA) and synthesized by Eurogentec (Seraing, Belgium). mRNA expression levels were calculated relative to the average of the housekeeping gene cyclophilin and further nomalized to the expression levels of the respective controls.

In vivo macrophage-to-feces reverse cholesterol transport studies
Experiments were performed as described before using thioglycollate-elicited primary peritoneal macrophages from C57BL/6J donor mice loaded in vitro with 50 μg/ml acetylated LDL and 3 μCi/ml [3H]cholesterol (Perkin Elmer, Boston, MA, USA) for 24 hours to become foam cells [29]. These were injected intraperitoneally into individually housed recipient mice. Counts in plasma collected 6, 24 and 48 hours after macrophage injection were assessed directly by liquid scintillation counting (Packard 1600CA Tri-carb, Packard, Meriden, CT, USA). Tracer uptake into the liver was

determined at 48 hours after injection following solubilization of the tissue (Solvable, Packard, Meriden, CT, USA) and radioactivity was determined by liquid scintillation counting [32]. Feces were collected over a 48-hour period, dried, weighed and ground. Aliquots were separated into neutral sterol and bile acid fractions [22] and radioactivity was determined by liquid scintillation counting. Counts were expressed relative to the administered dose.

Statistics
Statistical analyses were performed using GraphPad Prism (GraphPad software, San Diego, CA, USA). Data are presented as means ± SEM. Statistical differences between two groups were assessed using the Mann-Whitney U-test. P values below 0.05 were considered statistically significant.

Results

Systemic MTP inhibition decreases plasma triglycerides
To determine the effect of pharmacological inhibition of MTP activity C57BL/6J wild-type mice on chow diet were treated with the MTP inhibitor BMS-212122 for 2 weeks and compared to vehicle-treated controls. Plasma total cholesterol as well as triglyceride levels were decreased in MTP inhibitor-treated mice (each $p<0.001$, Figure 1A). Both free cholesterol as well as cholesterol ester contributed to the decrease of total cholesterol (each $p<0.001$, figure 1A). FPLC profiles indicated a decrease in HDL cholesterol (Figure 1B) and in VLDL triglycerides (Figure 1C) attributable to decreased hepatic VLDL and intestinal chylomicron production as a result of MTP inhibition (supplemental figure I).

Systemic MTP inhibition increases fecal, but not biliary sterol excretion
Body weight and liver weight were unchanged upon MTP inhibitor treatment (data not shown). Liver total and esterified cholesterol as well as triglyceride content was increased in MTP inhibitor receiveing mice compared to controls (each $p<0.001$, Figure 2A). There was a tendency towards an increase in free cholesterol (Figure 2A). Hepatic expression of the SREBP2 target genes HMGCoAR ($p<0.001$, Table 1) and LDLR ($p<0.05$, Table 1) were significantly lower in MTP inhibitor-treated mice, likely as a consequence of increased hepatic cholesterol accumulation. To explore if systemic MTP inhibition would also translate into changes in biliary sterol secretion, a 30 min continuous bile cannulation experiment was performed. Bile flow (data not shown), as well as biliary cholesterol, bile acid secretion (Figure 2B) and phospholipid secretion (data not shown) remained unchanged compared with controls. Next, the impact of MTP inhibition on fecal sterol excretion was investigated. A small decrease in fecal bile acid excretion was observed ($p<0.05$, Figure 2C), mainly in ω-muricholic, α-muricholic and deoxycholic acid (table 2). Fecal neutral sterol excretion, on the other hand, was significantly increased by 75% ($p<0.001$, Figure 2C,

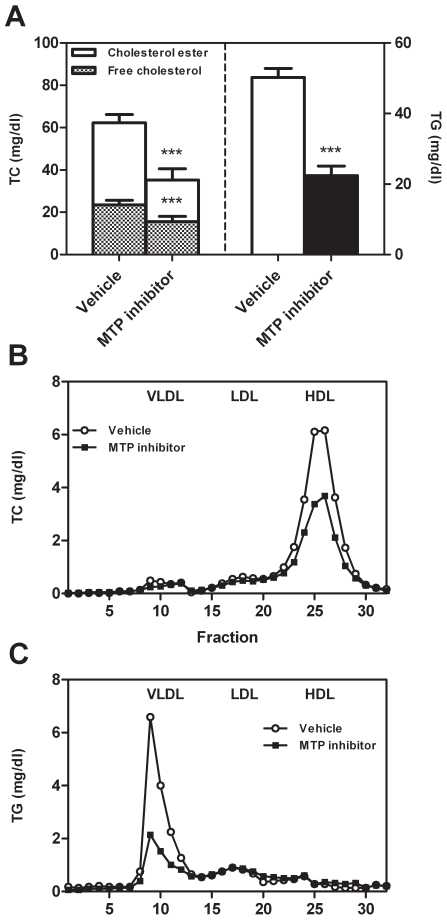

Figure 1: Impact of systemic MTP inhibition on plasma lipids. After 2 weeks of MTP inhibitor treatment mice were fasted for 4 hours and a blood sample was taken. (A) Plasma total cholesterol and triglyceride levels. Data are presented as means ± SEM. Statistically significant differences from control mice are indicated as ***p<0.001. FPLC profiles of pooled plasma samples for the distribution of (B) total cholesterol and (C) triglycerides over the different lipoprotein subclasses as indicated. At least n = 6 for each group.

table 2). This latter finding is likely attributable to a decrease in intestinal cholesterol absorption, since intestinal mRNA expression of NPC1L1 was 50% lower in MTP inhibitor receiving mice (Table 1), and patients with abetalipoproteinemia lacking intestinal MTP [4, 10] as well as intestine-specific MTP knockout mice exhibit decreased intestinal lipid absorption [14, 33]. Consistent with this hypothesis, subsequent determination of intestinal cholesterol absorption confirmed a 46% decrease in the mice treated with the pharmacological MTP inhibitor (48.1 ± 4.0 vs. 25.9 ± 1.7%, p<0.001).

Table 1: Hepatic and proximal intestinal mRNA expression in vehicle vs MTP inhibitor-treated wild-type mice and wild-type vs. liver-specific MTP knockout (L-Mttp-/-) mice.

	Vehicle	MTP inhibitor	Wild-type	L-*Mttp*$^{-/-}$
Hepatic mRNA expression				
Mtp	1.00 ± 0.04	0.96 ± 0.01	1.00 ± 0.06	0.13 ± 0.09***
Hmgcoar	1.00 ± 0.06	0.62 ± 0.05***	1.00 ± 0.12	0.35 ± 0.06***
Abca1	1.00 ± 0.08	0.99 ± 0.02	1.00 ± 0.06	0.88 ± 0.07
Sr-b1	1.00 ± 0.04	1.08 ± 0.01	1.00 ± 0.07	0.79 ± 0.05*
Ldlr	1.00 ± 0.05	0.78 ± 0.01*	1.00 ± 0.07	0.65 ± 0.06**
Small intestine mRNA expression				
Mtp	1.00 ± 0.06	1.21 ± 0.05	1.00 ± 0.05	0.74 ± 0.05*
Abca1	1.00 ± 0.21	0.63 ± 0.03	1.00 ± 0.18	0.61 ± 0.16
Abcg5	1.00 ± 0.09	0.65 ± 0.03**	1.00 ± 0.06	0.86 ± 0.12
Abcg8	1.00 ± 0.09	0.67 ± 0.03**	1.00 ± 0.08	0.98 ± 0.13
Npc1l1	1.00 ± 0.13	0.51 ± 0.03**	1.00 ± 0.13	1.24 ± 0.11
Ldlr	1.00 ± 0.10	1.01 ± 0.18	1.00 ± 0.11	0.95 ± 0.08

Mice were fasted for 4 h, livers were excised, weighed and stored at -80ºC until analysis. Individual genes are expressed as a percentage of the houskeeping gene cyclophilin and further nomalized to the expression levels of the respective controls. Data are presented as means ± SEM. At least n = 6 for each group. Statistically significant differences from control mice are indicated as *p<0.05, **p<0.01, ***p<0.001.

Systemic MTP inhibition increases macrophage-to-feces RCT

Next, a macrophage-to-feces RCT experiment was performed to determine, if the increase in mass fecal neutral sterol excretion upon systemic MTP inhibition would impact RCT. Plasma 3H-cholesterol levels were lower in MTP inhibitor treated mice at 24 (p<0.01) and 48 hours (p<0.05) after macrophage injection (Figure 3A), while tracer recovery within the liver was not affected (Figure 3B). However, systemic MTP inhibition significantly enhanced overall RCT reflected by a higher fecal excretion of the macrophage-derived 3H-cholesterol tracer (p<0.01, Figure 3C). This increase was mainly attributable to a 2-fold higher excretion of 3H-cholesterol within the fecal neutral sterol fraction (p<0.01, Figure 3C), although tracer recovery within the fecal bile acid fraction was also significantly higher (p<0.05, Figure 3C).

Hepatocyte-specific MTP deficiency decreases plasma triglycerides

To determine, if the metabolic effects observed in mice treated with a systemic MTP inhibitor were attributable to inhibition of hepatic or intestinal MTP, a similar set of experiments was performed in L-Mttp-/- mice compared with control littermates. Plasma total cholesterol and triglyceride levels were significantly reduced in L-Mttp-/- mice (each p<0.001, Figure 4A). The decrease in total cholesterol was attributable to a decrease of both free cholesterol and cholesterol ester (each p<0.001, Figure 4A). FPLC analysis revealed lower VLDL, LDL as well as HDL cholesterol levels in L-Mttp-/- mice (Figure 4B), while triglycerides within the apoB-containing lipoproteins VLDL and LDL were substantially reduced (Figure 4C).

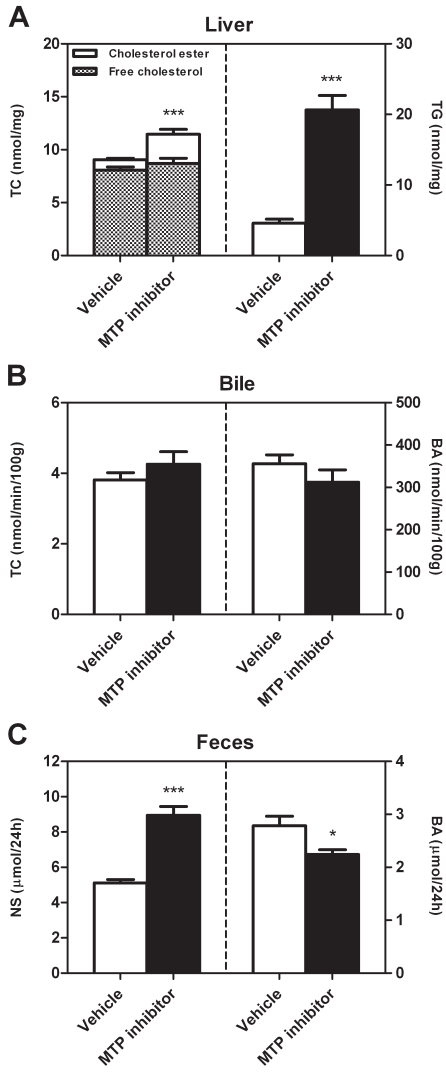

Figure 2: Impact of systemic MTP inhibition on liver lipids and biliary as well as fecal sterol excretion. (A) Hepatic contents of total cholesterol, free cholesterol, cholesterol ester and triglycerides. After 2 weeks of MTP inhibitor treatment mice were fasted for 4 hours, the liver was excised and cholesterol as well as triglyceride contents were determined as detailed in experimental procedures. (B) Biliary secretion rates of total cholesterol and bile acids. Continuous bile cannulation experiments for 30 min were performed after 2 weeks of inhibitor treatment as described in experimental procedures. (C) Mass fecal neutral sterol and bile acid excretion levels. After 2 weeks of inhibitor treatment feces were collected for a 24-hour period, processed and analyzed as detailed in experimental procedures. Data are presented as means ± SEM. At least n = 6 for each group. Statistically significant differences from control mice are indicated as *p<0.05, ***p<0.001.

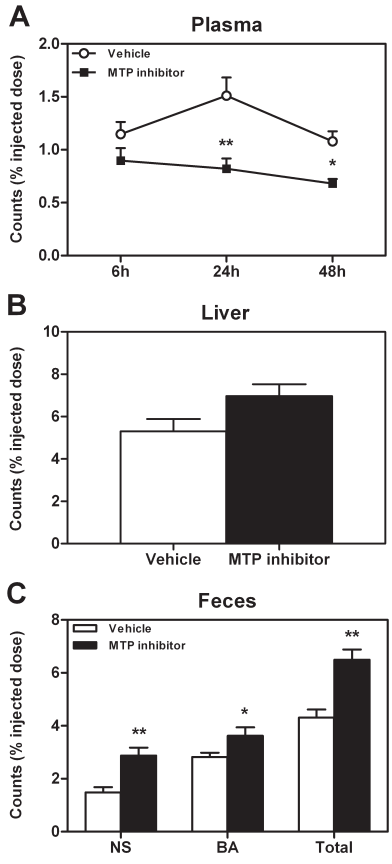

Figure 3: Impact of systemic MTP inhibition on macrophage-to-feces RCT. Following 2 weeks of MTP inhibitor treatment an in vivo macrophage-to-feces RCT experiment was performed as detailed in experimental procedures. (A) cholesterol tracer appearance in plasma 6, 24 and 48 hours after macrophage administration. (B) cholesterol tracer recovery within liver 48 hours after macrophage injection. (C) cholesterol tracer appearance in feces collected continuously from 0 to 48 hours after macrophage injection. Data are presented as means ± SEM. At least n = 6 for each group. Statistically significant differences from the control group are indicated as *p<0.05, **p<0.01.

Heptocyte-specific MTP deficiency does not impact biliary or fecal sterol excretion

Body weight as well as liver weight of L-Mttp-/- mice was unchanged compared to wild-type controls (data not shown). Consistent with the results obtained in mice treated with the systemic MTP inhibitor, liver total cholesterol and cholesterol ester content was increased in L-Mttp-/- mice (p<0.001, Figure 5A). Also in the L-Mttp-/- mice, hepatic mRNA expression of the SREBP2 target genes HMGCoAR (p<0.001, Table 1) and LDLR (p<0.01, Table 1) was significantly lower, likely as a consequence of hepatic cholesterol accumulation. Hepatic triglyceride content was increased 5-fold (p<0.001, Figure 5A). Next we performed a 30 minute continuous bile can-

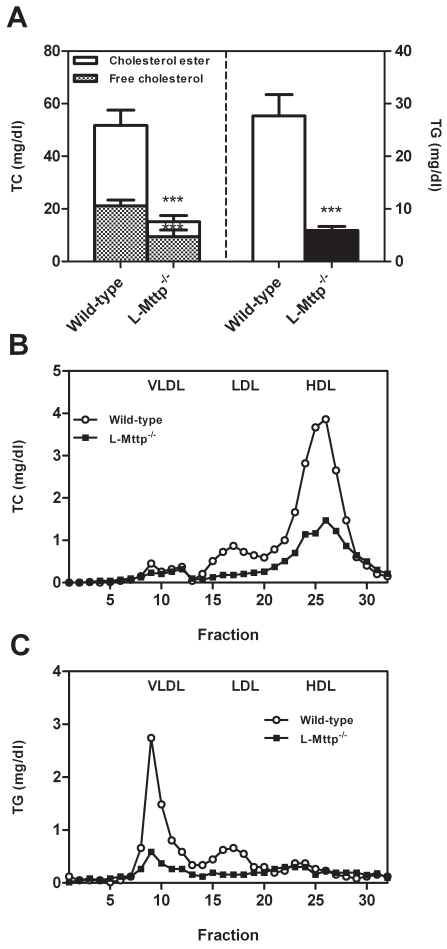

Figure 4: Impact of hepatocyte-specific MTP deficiency (L-Mttp-/-) on plasma lipid levels and distribution. L-Mttp-/- mice were fasted for 4 hours and a blood sample was taken. (A) Plasma total cholesterol and triglyceride levels. Data are presented as means ± SEM. Statistically significant differences from control mice are indicated as ***p<0.001. FPLC profiles of pooled plasma samples for the distribution of (B) total cholesterol and (C) triglycerides over the different lipoprotein subclasses as indicated. At least n = 6 for each group.

nulation experiment to determine the impact of hepatocyte-specific MTP deficiency on biliary secretion rates. Bile flow was unchanged compared to wild-type controls (data not shown) as were the biliary secretion rates of cholesterol (Figure 5B), bile acids (Figure 5B) and phospholipids (data not shown). In contrast to systemic MTP inhibition, eliminating MTP in the liver alone did not affect the fecal excretion of either neutral sterols or bile acids (Figure 5C, table 3). Consistent with these findings, in the L-Mttp-/- model no changes in the intestinal expression of NPC1L1 were observed (Table 1) and also experimentally measured intestinal cholesterol absorption rates were identical in wild-type and L-Mttp-/- mice (52.2 ± 5.3 vs. 52.1 ± 5.5%, n.s.).

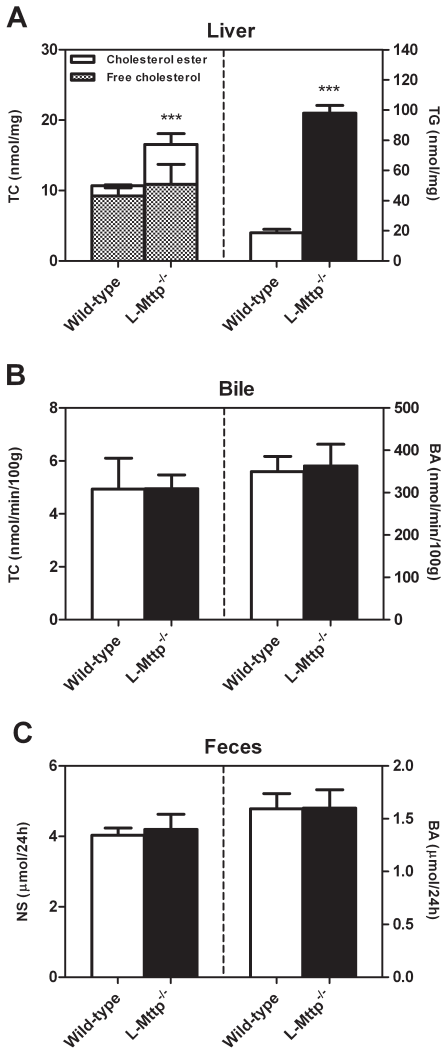

Figure 5: Impact of hepatocyte-specific MTP deficiency (L-Mttp-/-) on liver lipids and biliary as well as fecal sterol excretion. (A) Hepatic contents of total cholesterol, free cholesterol, cholesterol ester and triglycerides. L-Mttp-/- mice were fasted for 4 hours, the liver was excised and cholesterol as well as triglyceride contents were determined as detailed in experimental procedures. (B) Biliary secretion rates of total cholesterol and bile acids. Continuous bile cannulation experiments for 30 min were performed in L-Mttp-/- mice as described in experimental procedures. (C) Mass fecal neutral sterol and bile acid excretion levels. Feces from L-Mttp-/- mice were collected for a 24-hour period, processed and analyzed as detailed in experimental procedures. Data are presented as means ± SEM. At least n = 6 for each group. Statistically significant differences from control mice are indicated as ***p<0.001.

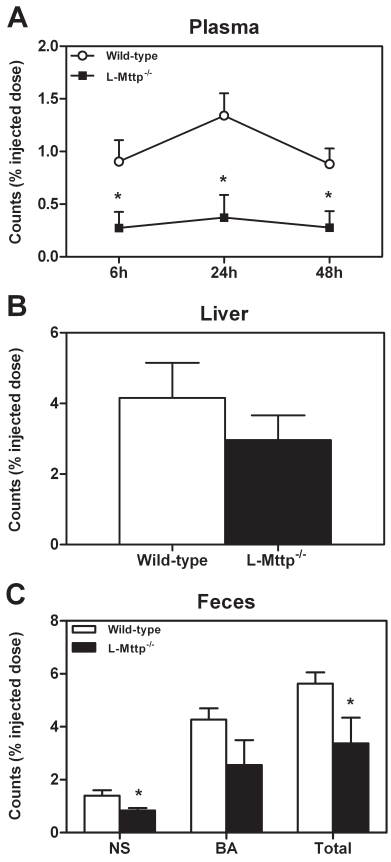

Figure 6: Impact of hepatocyte-specific MTP deficiency (L-Mttp-/-) on macrophage-to-feces RCT. An in vivo macrophage-to-feces RCT experiment was performed in L-Mttp-/- mice as detailed in experimental procedures. (A) cholesterol tracer appearance in plasma 6, 24 and 48 hours after macrophage administration. (B) cholesterol tracer recovery within liver 48 hours after macrophage injection. (C) cholesterol tracer appearance in feces collected continuously from 0 to 48 hours after macrophage injection. Data are presented as means ± SEM. At least n = 6 for each group. Statistically significant differences from the control group are indicated as *p<0.05.

Hepatocyte-specific MTP deficiency decreases macrophage-to-feces RCT

Next we investigated the contribution of hepatic MTP expression to the changes in RCT observed with the systemic MTP inhibitor and performed a macrophage-to-feces RCT experiment in the hepatocyte-specific MTP-deficient mice. Plasma 3H-cholesterol levels were lower in L-Mttp-/- at 6, 24 and 48 hours after macrophage injection (each $p<0.05$, Figure 6A), while tracer recovery within the liver at 48 hours after macrophage injection was unchanged (Figure 6B). Most importantly and in contrast to the results with systemic MTP inhibition, the total excretion of 3H-cholesterol originating from macrophages into the feces was decreased in L-Mttp-/- mice ($p<0.05$, Figure 6C). This was mainly attributable to a significantly lower recovery of

Table 2: Fecal excretion of neutral sterol and bile acid species in vehicle- and MTP inhibitor-treated wild-type mice.

	Vehicle	MTP inhibitor
Fecal output (g/24h)	0.85 ± 0.07	0.87 ± 0.07
Neutral sterol species in feces		
Cholesterol (nmol/24h)	3799.20 ± 213.87	6318.96 ± 469.60***
Dihydrocholesterol (nmol/24h)	385.76 ± 12.66	417.26 ± 17.62
Coprostanol (nmol/24h)	930.97 ± 95.82	2208.34 ± 490.14*
Bile acid species in feces		
Allocholic acid (nmol/24h)	68.64 ± 7.02	59.97 ± 4.49
α-Muricholic acid (nmol/24h)	153.77 ± 12.85	97.95 ± 3.26***
β-Muricholic acid (nmol/24h)	206.10 ± 17.19	190.64 ± 7.24
ω-Muricholic acid (nmol/24h)	894.72 ± 67.29	713.17 ± 42.57*
Cholic acid (nmol/24h)	199.34 ± 18.54	169.75 ± 16.01
Deoxycholic acid (nmol/24h)	1154.51 ± 90.40	934.82 ± 40.57*
Hyodeoxycholic acid (nmol/24h)	107.31 ± 7.81	74.76 ± 7.71*

Feces were collected over a 24h period. Data are presented as means ± SEM. At least n = 6 for each group. Statistically significant differences from control mice are indicated as *p<0.05, ***p<0.001.

Table 3: Fecal excretion of neutral sterol and bile acid species in wild-type and L-Mttp-/- mice.

	Wild-type	L-*Mttp*$^{-/-}$
Fecal output (g/24h)	0.76 ± 0.07	0.74 ± 0.03
Neutral sterol species in feces		
Cholesterol (nmol/24h)	3435.09 ± 167.02	3739.75 ± 399.07
Dihydrocholesterol (nmol/24h)	219.52 ± 15.57	241.32 ± 21.22
Coprostanol (nmol/24h)	378.21 ± 145.86	213.99 ± 90.91
Bile acid species in feces		
Allocholic acid (nmol/24h)	23.41 ± 1.85	31.31 ± 5.64
α-Muricholic acid (nmol/24h)	n.d.	n.d.
β-Muricholic acid (nmol/24h)	218.83 ± 30.02	241.86 ± 53.44
ω-Muricholic acid (nmol/24h)	552.03 ± 59.69	483.89 ± 69.23
Cholic acid (nmol/24h)	138.43 ± 16.72	175.44 ± 34.75
Deoxycholic acid (nmol/24h)	599.22 ± 46.52	624.25 ± 43.97
Hyodeoxycholic acid (nmol/24h)	57.55 ± 5.17	41.07 ± 3.36*

Feces were collected over a 24h period. Data are presented as means ± SEM. At least n = 6 for each group. Statistically significant differences from control mice are indicated as *p<0.05. n.d. is not detected.

the cholesterol tracer within the fecal neutral sterol fraction (p<0.05, Figure 6C) and there was a trend towards lower tracer recovery within the bile acid fraction as well (p=0.12, Figure 6C).

Discussion

This study investigated the consequences of differentially lowering MTP activity systemically with a pharmacological inhibitor or genetically in the liver only on cholesterol metabolism with a specific focus on RCT. Our data demonstrate that decreasing intestinal and hepatic secretion of apoB-containing lipoproteins via inhibition of MTP increases RCT, while the absence of hepatocyte MTP expression has the opposite effect. These results indicate on the one hand that hepatic secretion of RCT-relevant cholesterol within apoB-containing lipoproteins contributes to RCT and on the other that upon pharmacological MTP inhibition the decrease in intestinal cholesterol absorption has a relatively larger effect on RCT than the decrease in VLDL production.

MTP expression in the intestine is essential for chylomicron formation [4, 5, 10]. Genetic absence of intestinal MTP consequently results in malabsorption of lipids with all its adverse effects such as deficiency in fat-soluble vitamins [4, 10]. In the present study, a systemic but not a hepatocyte-specific deficiency of MTP activity resulted in decreased intestinal cholesterol absorption and increased fecal sterol excretion suggesting that the effect of chemical MTP inhibition on RCT is mainly mediated by the intestine by decreasing cholesterol absorption. Similar effects on RCT have been observed previously with the use of the NPC1L1 inhibitor ezetimibe [25-27]. These previous studies consistently demonstrated that targetin intestinal sterol absorption, and thereby the last step of RCT, is an effective means of increasing the fecal excretion of macrophage-derived cholesterol [25-27].

The major part of plasma cholesterol within apoB-containing lipoproteins originates from hepatic secretion of VLDL [34]. Thereby, hepatocyte MTP is considered to have a prime relevance for atherosclerotic CVD. Consequently, a number of therapeutic strategies were developed aiming to substantially decrease hepatic VLDL secretion, among these antisense approaches targeting MTP as well as apoB [17, 35]. However, only antisense therapeutics against apoB have been evaluated in human clinical trials [35]. These have proven to possess a high efficacy to decrease circulating levels of pro-atherogenic lipoproteins that would be expected on the basis of epidemiological data to translate into a substantial reduction in cardiovascular risk [35]. On the other hand, effects on HDL, a protective lipoprotein subclass for CVD development, have not been addressed. Specifically the functionality of HDL particles represents an emerging concept in cardiovascular research [36, 37]. A prime established atheroprotective function of HDL is to mediate RCT [20, 38]. In the present study we demonstrate that a hepatocyte-specific lack in MTP decreases RCT, which would be expected to counteract some of the beneficial effects of decreasing hepatic VLDL production.

How a decrease specifically in hepatic VLDL production might cause a decrease in RCT is not immediately apparent. There are several possibilities for the rerouting of hepatic cholesterol when MTP activity is lacking. One would be a change in bile acid synthesis, which can be excluded on the basis of unchanged fecal bile acid excretion

in the L-Mttp-/- mice. Another possibility would be a change in biliary cholesterol secretion, a pathway of high relevance for macrophage-to-feces RCT [39]. However, the biliary secretion of cholesterol remained unaltered in all the models with decreased MTP activity investigated in our study, which also makes changes in this route unlikely. A third possibility, however, likely inversely linked to the biliary secretion pathway [22], could be an increased resecretion via ABCA1. Also this pathway does not appear causative to explain our results, since, in addition to unchanged biliary sterol secretion, a decrease in HDL cholesterol and not an increase was noted in response to absent hepatic MTP and no apparent changes in ABCA1 expression were observed.

These considerations leave the most prominent phenotype, the decrease in hepatic VLDL production itself as a likely explanation. In previous work we demonstrated that following hepatic uptake HDL-derived cholesterol can be resecreted within VLDL [24]. In another study, where we tested the relevance of the biliary pathway for RCT, we found that, although biliary secretion plays the predominant role for RCT, still around 25% of macrophage-derived counts in the fecal neutral sterol fraction were excreted in Abcb4 knockout mice that have absent biliary cholesterol secretion [39]. How can these data be integrated into a model? Recently, an intestinal pathway of cholesterol excretion from the body is increasingly gaining interest, the concept of transintestinal cholesterol excretion (TICE) [31, 40, 41]. For TICE a number of key questions have not been answered, yet, including the lipoprotein substrates donating cholesterol for this pathway, their cellular uptake, the trafficking routes within the enterocyte and the apical transporters mediating final excretion into the intestinal lumen [42]. Regarding the apical transporters, only for ABCG5/G8 a significant impact was shown, but still models with absent TICE are lacking, which complicates studying the relevance of this pathway [31, 42]. Although formally HDL has not been excluded as a substrate for TICE, present data strongly suggest that apoB-containing lipoproteins serve as a main cholesterol donor particle for the pathway [43, 44]. Conceivably, the decrease in RCT in the L-Mttp-/- mice could therefore be explained by a fraction of RCT-relevant cholesterol entering the liver on HDL, then being resecreted within VLDL and subsequently taken up by the intestine for excretion from the body via TICE. Such a concept would actually be consistent with results that suggested that the intestine contributes to RCT based on data obtained in short-term RCT experiments employing a bile-diverted mouse model [45], while yet another study supported the view that the major pathway for RCT occurs via biliary secretion [46]. However, we would also like to point out that all these data, including the present study, were generated in mice, a predominant HDL species. The conclusions might therefore not be directly translatable into the human system.

In summary, systemic pharmacological inhibition and liver-specific deficiency in MTP activity have differential effects on cholesterol metabolism and RCT with pharmacological inhibition increasing and liver-specific absence of MTP decreasing RCT. These results indicate that the intestine contributes to RCT in two different ways, by absorbing more or less cholesterol secreted via the bile and by excreting RCT-

relevant cholesterol that is secreted by hepatocytes within apoB-containing lipoproteins. In a therapeutic perspective our data suggest that systemic or intestinal MTP inhibition might be preferrable to not only lower plasma levels of proatherogenic apoB-containing lipoproteins but to also beneficially impact RCT, a major function of the anti-atherogenic HDL lipoprotein subclass.

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Supplemental material chapter 4

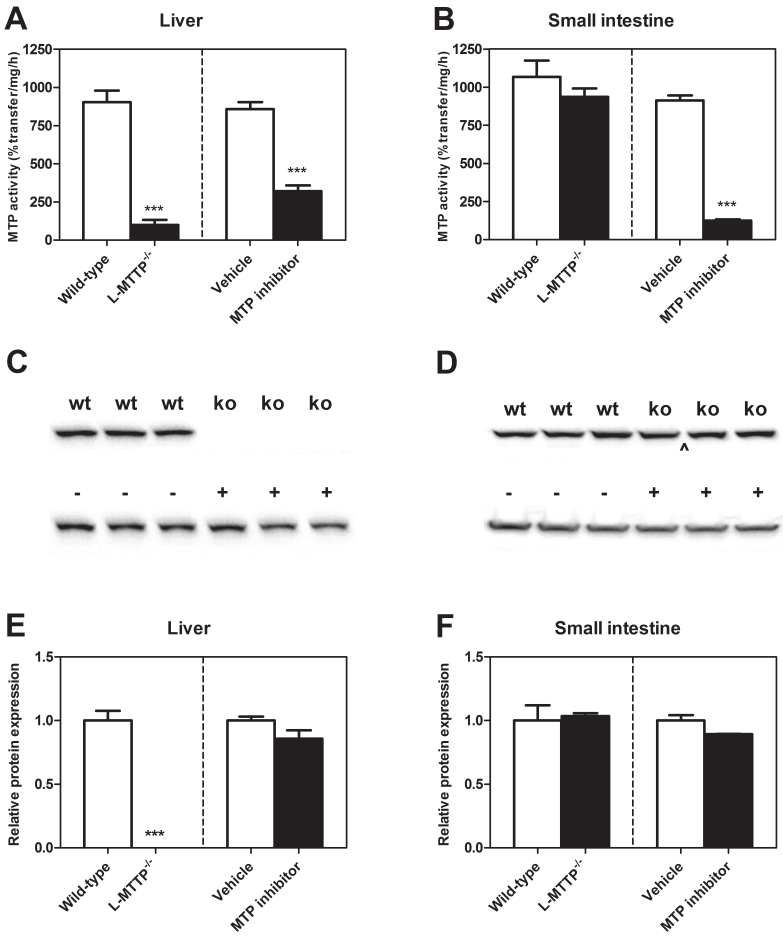
Supplemental Materials and Methods

VLDL production studies
VLDL production was essentially determined as described before [1]. Mice were fasted for 4 hours, injected i.p. with poloxamer 407 (1000 mg/kg body weight) and blood samples were drawn at 0, 30, 60 and 180 minutes after injection. Plasma triglycerides were measured enzymatically (Diasys, Holzheim, Germany) and the triglyceride production rate was calculated as described [1].

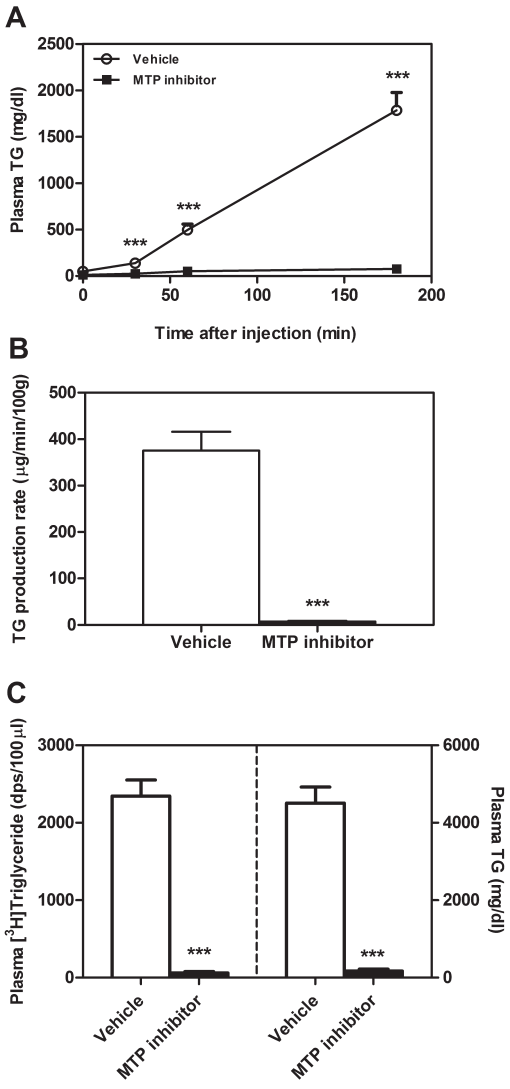
Chylomicron production studies
Mice were fasted for 4 hours and then injected i.p. with poloxamer 407 (1000 mg/kg body weight). Immediately, 150 µl olive oil containing 2 µCi 3[H]triolein was administered via gavage and a blood sample was drawn at 3 hours. Counts in plasma were assessed directly by liquid scintillation counting (Packard 1600CA Tri-carb, Packard, Meriden, CT). Plasma triglycerides were measured enzymatically (Diasys, Holzheim, Germany).

MTP activity measurements
MTP activity was measured in liver and proximal small intestine using a fluorigenic assay essentially as described before [1].

Western blotting
Western blots for MTP were carried out as described before on total liver and proximal small intestine homogenates [2]. The bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL) was used to determine protein concentrations. 50 µg protein was resolved by SDS-PAGE electrophoresis and blotted onto nitrocellulose. MTP was visualized using a mouse MTP antibody (BD Biosciences, Franklin Lakes, NJ), followed by a HRP-conjugated secondary antibody. HRP was detected using chemiluminescence (ECL, GE Healthcare, Piscataway, NJ). Densitometry analysis of the bands was performed on Western blots using ImageJ software (National Institutes of Health, Bethesda, MD).



Supplemental figure I: Impact of hepatocyte-specific MTP deficiency (L-Mttp-/-) and pharmacological MTP inhibition on MTP protein expression as well as MTP activity in liver and small intestine. L-Mttp-/- and MTP inhibitor-treated mice were fasted for 4 hours before liver and proximal small intestine were excised and stored at -80°C until analysis. MTP activity and protein expression were determined as detailed in Supplemental Materials and Methods. (A) MTP activity in liver. (B) MTP activity in small intestine. (C) MTP protein expression and (E) quantification in liver. (D) MTP protein expression and (F) quantification in small instestine. Data are presented as means ± SEM. At least n = 6 for each group (A and B), n = 3 for each group (C-F). For Western blot (C and D) wild-type control, L-Mttp-/-, vehicle treated and pharmacologically treated mice are indicated as wt, ko, - and + respectively. ^ indicates were the marker was cut out. Statistically significant differences from the control group are indicated as ***p<0.001.



Supplemental figure II: Impact of pharmacological MTP inhibition on VLDL and chylomicron production. MTP inhibitor-treated mice were fasted for 4 hours and poloxamer 407 was injected i.p. (A) plasma triglyceride levels at 0, 30, 60 and 180 minutes after poloxamer 407 administration. (B) calculated triglyceride production rates. To determine chylomicron production mice were fasted for 4 hours, poloxamer 407 was injected i.p. and 150 µl olive oil containing 2 µCi 3[H]triolein was administered via gavage. (C) Plasma 3[H]triglyceride tracer recovery and plasma triglyceride concentrations 3 hours after gavage. Data are presented as means ± SEM. At least n = 6 for each group. Statistically significant differences from the control group are indicated as *p<0.05, ***p<0.001.

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Chapter 5

Hepatic ABCG5/G8 overexpression substantially increases biliary cholesterol secretion but does not impact in vivo macrophage-to-feces RCT

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Abstract

Biliary cholesterol secretion is important for reverse cholesterol transport (RCT). ABCG5/G8 contribute most cholesterol mass secretion into bile. We investigated the impact of hepatic ABCG5/G8 on cholesterol metabolism and RCT.
Biliary and fecal sterol excretion (FSE) as well as RCT were determined using wild-type controls, Abcg8 knockout mice, Abcg8 knockouts with adenovirus-mediated hepatocyte-specific Abcg8 reinstitution and hepatic Abcg5/g8 overexpression in wild-types.
In Abcg8 knockouts, biliary cholesterol secretion was decreased by 75% ($p<0.001$), while mass FSE and RCT were unchanged. Hepatic reinstitution of Abcg8 increased biliary cholesterol secretion 5-fold ($p<0.001$) without changing FSE or overall RCT. Overexpression of both ABCG5/G8 elevated biliary cholesterol secretion 5-fold and doubled FSE ($p<0.001$) without affecting overall RCT.
ABCG5/G8 mediate mass biliary cholesterol secretion but not from a RCT-relevant pool. Intervention strategies aiming at increasing hepatic Abcg5/g8 expression for enhancing RCT are not likely to be successful.

Introduction

Reverse cholesterol transport (RCT) is a multistep atheroprotective pathway comprising cholesterol efflux from peripheral cells towards HDL, uptake by the liver, secretion into the bile and excretion via the feces [1]. The obligatory heterodimerizing half-transporters ATP-binding cassette subfamily G members 5 and 8 (ABCG5/G8) are expressed in liver and small intestine [2]. In the small intestine, ABCG5/G8 resecrete absorbed plant sterols and cholesterol back into the intestinal lumen thereby decreasing intestinal sterol absorption [3]. In the liver, ABCG5/G8 is the sterol transport system contributing quantitatively the highest amount of mass cholesterol secretion into bile, approximately 75% [4, 5]. Biliary cholesterol secretion has major relevance for functional RCT [6]. Since the specific importance of hepatic ABCG5/G8 in this process has not been directly investigated, the aim of the present study was to determine the impact of liver ABCG5/G8 expression on in vivo macrophage-to-feces RCT.

Materials and Methods

Animals
C57BL/6J wild-type (Charles River, Sulzfeld, Germany) and Abcg8 knockout mice [7] were housed in 21°C temperature-controlled rooms with alternating 12 hour periods of light and dark and ad libitum access to water and mouse chow diet (Arie Blok, Woerden, The Netherlands). Animal experiments were performed in accordance with national laws and approved by the responsible ethics committee of the University of Groningen.

Analysis of plasma lipids
Blood was obtained by cardiac puncture under anesthesia after a 4-hour fasting period. Plasma aliquots were stored at -80°C until analysis. Commercially available reagents were used to measure plasma free cholesterol (Diasys, Holzheim, Germany) total cholesterol and triglycerides (Roche Diagnostics, Mannheim, Germany). These enzymatic reragents do not differentiate cholesterol from phytosterols. Please note, that a relatively high percentage (~40%) of sterols in plasma of chow-fed Abcg8 ko mice is actually phytosterol [7].

Adenovirus administration
Recombinant adenoviruses were constructed and propagated as detailed previously [8]. Mice were injected with 1 x 1011 particles/mouse of AdNull, AdAbcg8 or AdAbcg5/g8 (0.5 x 1011 each). In vivo RCT studies were performed between day 3 and day 5 after injection of the respective adenoviruses, a time frame when high and stable expression is achieved. All other experiments were carried out on day 4 after adenovirus injection.

Bile collection and composition analysis
The gallbladder was cannulated, bile collected for 30 minutes and production was determined gravimetrically as described [4]. Biliary cholesterol and bile acid concentrations were determined by gas chromatography-mass spectrometry and the respective biliary secretion rates calculated [9].

Analysis of fecal neutral sterol and bile acid excretion
Feces of individually housed mice were collected for 24h. Fecal samples were dried, weighed and ground. Aliquots were used for the determination of neutral sterols and bile acids by gas-liquid chromatography as described [4].

In vivo macrophage-to-feces reverse cholesterol transport studies
Thioglycollate-elicited primary peritoneal macrophages from C57BL/6J donor mice were loaded in vitro with acetylated LDL and [3H]cholesterol (Perkin Elmer, Boston, MA, USA) for 24 hours to become foam cells and were following equilibration injected intraperitoneally into individually housed recipient mice [10]. Tracer recovery in plasma, liver and feces collected for 48h was determined by liquid scintillation counting (Packard 1600CA Tri-carb, Packard, Meriden, CT, USA) [9]. Feces aliquots were separated into neutral sterol and bile acid fractions [9]. Counts were expressed relative to the administered dose.

Statistics
Statistical analyses were performed using Prism (GraphPad software, San Diego, CA, USA). All data are presented as median with 5th and 95th percentiles. Statistical differences between two groups were assessed using the Mann-Whitney U-test, P values below 0.05 considered statistically significant.

Results

Despite substantially decreased biliary cholesterol secretion total macrophage-to-feces RCT is unchanged in Abcg8 ko mice

To determine the impact of ABCG5/G8 on in vivo RCT we first compared wild-type with Abcg8 ko mice. Plasma total cholesterol as well as triglyceride levels were increased in Abcg8 ko mice (each $p<0.001$, Figure 1A). A 30 minute continuous bile cannulation experiment showed a substantial decrease in biliary cholesterol and also a decrease in bile acid secretion in Abcg8 ko mice ($p<0.001$ and $p<0.01$ respectively, Figure 1B). There was no change in fecal neutral sterol excretion (Figure 1C) but a small increase in fecal bile acid excretion in Abcg8 ko mice ($p<0.05$, Figure 1C). Next, we performed a macrophage-to-feces RCT experiment to determine if the decrease in biliary cholesterol secretion would impact RCT. Plasma 3H-cholesterol levels were unchanged (Figure 2A) while tracer recovery within the liver was lower ($p<0.05$, Figure 2B). There was a small decrease in fecal loss of 3H-tracer within neutral sterols ($p<0.05$), however, since there was no difference in 3H-tracer within the fecal bile acid fraction this did not translate into a difference in overall RCT (Figure 2C).

Hepatic reinstitution of ABCG8 in Abcg8 ko mice substantially increases biliary cholesterol secretion but does not impact total macrophage-to-feces RCT

To assess the effect of hepatic Abcg8 reinstitution on in vivo macrophage-to-feces RCT, Abcg8 ko mice were injected with either empty control adenovirus AdNull or with an adenovirus expressing Abcg8. There was a small decrease in plasma total cholesterol ($p<0.05$, Figure 1D) but no difference in plasma triglyceride levels (Figure 1D). Biliary bile acid secretion was unchanged (Figure 1E), however, mass biliary cholesterol secretion was increased 4-fold in Abcg8 knockouts with hepatocyte-specific Abcg8 expression compared with AdNull-injected Abcg8 ko controls ($p<0.001$, Figure 1E). Despite the 4-fold increase in biliary cholesterol secretion there was no difference in fecal neutral sterol and bile acid excretion (Figure 1F). Next, a macrophage-to-feces RCT experiment was performed. Plasma 3H-cholesterol was unchanged (Figure 2D) while tracer recovery within the liver was increased ($p<0.05$, Figure 2E). There was no impact on overall RCT despite a small increase in 3H-tracer recovery within the fecal neutral sterol fraction ($p<0.05$, Figure 2F).

Hepatic overexpression of ABCG5/G8 substantially increases biliary cholesterol secretion but does not impact total macrophage-to-feces RCT in wild-type mice

To assess the effect of hepatic Abcg5/g8 overexpression on in vivo macrophage-to-feces RCT, wild-type mice were injected either with empty control adenovirus AdNull or with adenoviruses expressing both Abcg5 and Abcg8. Comparable to hepatic Abcg8 reinstitution, there was a small decrease in plasma total cholesterol ($p<0.05$, Figure 1G) but no difference in plasma triglyceride levels (Figure 1G). Biliary bile acid secretion was unchanged (Figure 1H), while biliary cholesterol secretion was 4-fold increased ($p<0.001$, Figure 1H). This substantial increase in biliary cholesterol

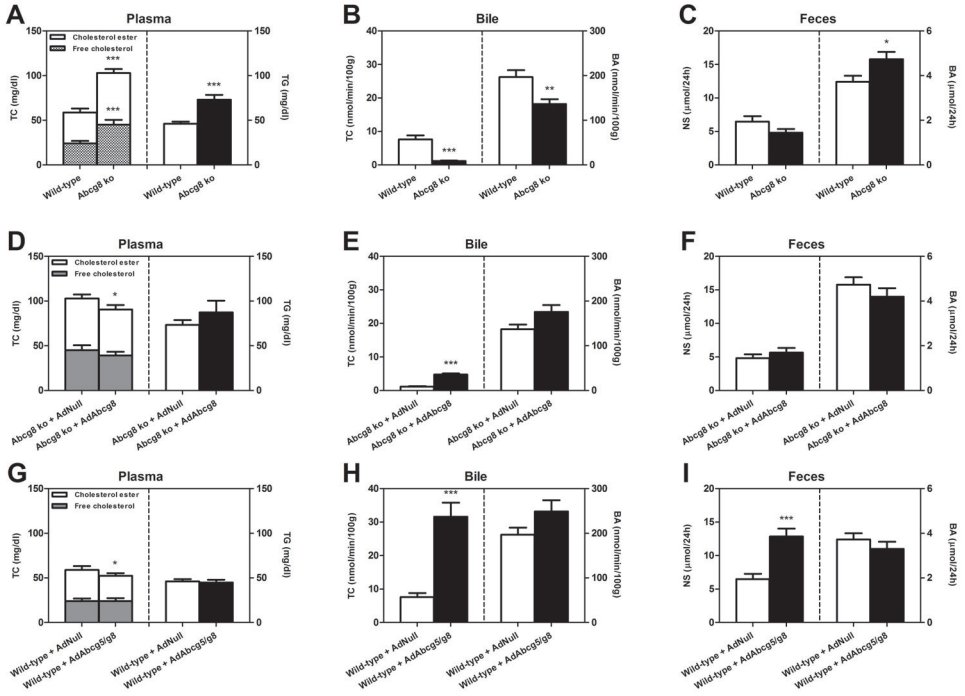

Figure 1: Impact of ABCG8 deficiency, hepatic ABCG8 reinstitution and hepatic ABCG5/G8 overexpression on plasma lipids, biliary sterol secretion and fecal sterol excretion. (A, D, G) plasma total cholesterol and triglyceride levels. (B, E, H) mass biliary secretion rates of total cholesterol and bile acids. (C, F, I) mass fecal neutral sterol and bile acid excretion. Data are presented as means ± SEM. At least n = 6 for each group. Statistically significant differences from the respective control group are indicated as *p<0.05, **p<0.01, ***p<0.001.

secretion translated into a 2-fold higher mass fecal neutral sterol excretion (p<0.001, Figure 1I) while fecal bile acid excretion remained unchanged (Figure 1I). In the RCT experiment, plasma (Figure 2F) and liver tracer recovery were unaltered (Figure 2H). There was also no impact on overall RCT despite a slight increase in 3H-tracer recovery within the fecal neutral sterol fraction (p<0.05, Figure 2I).

Discussion

Combined the results of the present study indicate that the hepatic expression level of ABCG5/G8 has only minor relevance for macrophage-to-feces RCT although these transporters contribute the major part to mass biliary cholesterol secretion. Specifically, despite discrete changes in tracer excretion in the fecal neutral sterol fraction, overall RCT remained essentially unaltered over a wide range of hepatic Abcg5/g8 expression levels spanning from knockouts, with a 75% reduction in biliary choles-

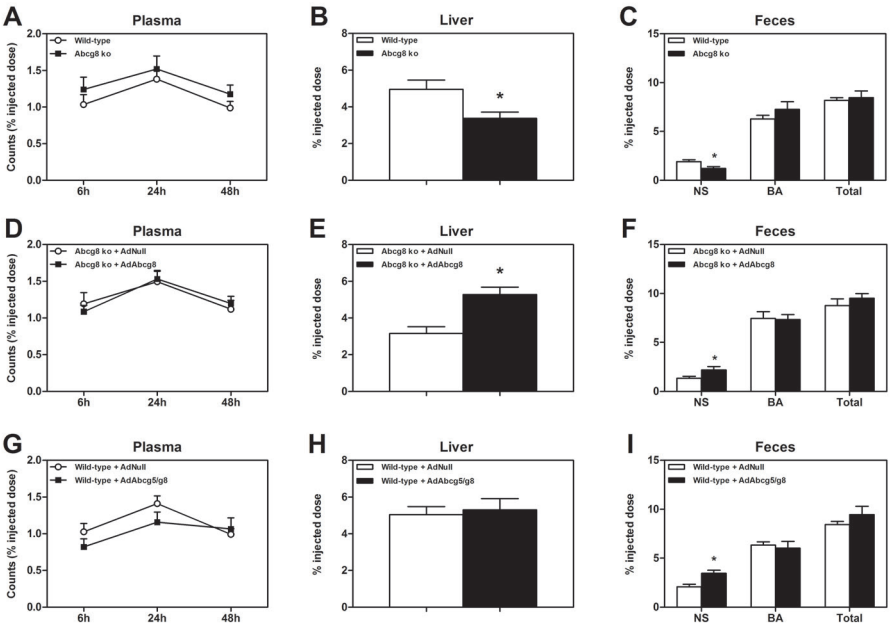

Figure 2: Impact of ABCG8 deficiency, hepatic ABCG8 reinstitution and hepatic ABCG5/G8 overexpression on macrophage-to-feces RCT. (A, D, G) cholesterol tracer appearance in plasma 6, 24 and 48 hours after macrophage injection. (B, E, H) cholesterol tracer recovery within the liver 48 hours following macrophage injection. (C, F, I) cholesterol tracer recovery in feces collected continuously from 0 to 48 hours after macrophage injection. Data are presented as means ± SEM. At least n = 6 for each group. Statistically significant differences from the control group are indicated as *p<0.05.

terol secretion, to high level overexpression resulting in 4-fold increased biliary cholesterol secretion rates. How can the dissociation between mass biliary cholesterol secretion and RCT be explained? One possible interpretation is that the RCT-relevant cholesterol is secreted from a hepatic cholesterol pool that is somehow less accessible to ABCG5/G8. On the other hand, the HDL receptor SR-BI seems more directly important for functional RCT as indicated by gain- and loss-of-function studies [11]. In addition, we previously demonstrated that abolishing SR-BI expression in Abcg5 knockout mice decreased RCT by 50% [4], further strengthening the importance of SR-BI over Abcg5/g8 for this pathway.

Another important factor in the regulation of cholesterol metabolism and fecal sterol output is the intestine. The intestinal compartment impacts fecal sterol excretion in two ways, via cholesterol absoprtion and via transintestinal cholesterol excretion (TICE) [12]. Manipulating the last step of RCT in the intestine, by e.g. abolishing cholesterol absorption with ezetimibe, increases fecal neutral sterol excretion together with RCT [10]. Previous results from liver-specific Abcg5/g8 transgenic mice showed that biliary cholesterol secretion was increased [13], but fecal neutral sterol excretion only increased when cholesterol absorption was blocked with ezetimibe

[13, 14]. Consistently, atherosclerotic lesion development in these liver-specific Abcg5/g8 transgenic mice changed only in the context of inhibiting intestinal cholesterol absorption with ezetimibe [13, 14]. These data suggest that also in our study intestinal absorption of macrophage-derived 3H-cholesterol could be limiting the 3H-neutral sterol contribution to RCT. Alternatively, TICE could be altered, since absence of Abcg5 was shown previously to contribute to TICE [15]. However, more work is required to delineate this possibility. Overall, our present results indicate that for the role of Abcg5/g8 in RCT hepatic expression might be less relevant than intestinal expression. In line with these data it was also shown that raising specifically the intestinal expression of Abcg5/g8 via LXR activation increases RCT [16, 17] and subsequently decreases atherosclerosis [17].

We feel that it is important to point out that for certain parameters the number of mice used in our studies might have been too low to detect significant differences previously reported in experiments from other groups. Specifically, this relates to mass fecal neutral sterol excretion, where previous work indicated a significant decrease in chow-fed Abcg5/g8 knockout mice [18], while in our study this trend was not significant. In addition and complementary to our present work, a recent study reported the generation of hepatocyte- and enterocyte-specific Abcg5/g8 knockout mice [19]. In terms of cholesterol metabolism, mass biliary cholesterol levels were decreased in the hepatocyte-specific ko, while fecal cholesterol excretion was unaltered among the models. The fecal appearance of i.v. administered 3H-cholesterol in intralipid was decreased in both, hepatocyte- and enterocyte-specific Abcg5/g8 ko; macrophage-to-feces RCT studies were not performed [19].

In summary, our data demonstrate that hepatic Abcg5/g8 expression is of minor relevance for functional in vivo RCT. These results not only offer a mechanistic explanation for previously obtained data from atherosclerosis studies in the context of manipulating Abcg5/g8 expression, but also indicate that intervention strategies aiming at increasing hepatic Abcg5/g8 expression for enhancing RCT are not likely to be successful.

Acknowledgments

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Chapter 6

General Discussion

Arne Dikkers

The importance of understanding reverse cholesterol transport

Cholesterol is a key structural component of animal cell membranes. However, cholesterol levels need to be tightly regulated as the accumulation of excessive cholesterol within the body can promote the development of major disease complexes such as atherosclerotic cardiovascular disease (CVD). According to the World Health Organization the number one cause of death throughout the world is cardiovascular disease [54]. Large population studies have shown that low density lipoprotein (LDL) cholesterol levels are directly correlated with clinical events resulting from CVD, whereas levels of high density lipoprotein (HDL) cholesterol as well as the major HDL apolipoprotein constituent apolipoprotein A-I (apoA-I) have an inverse correlation with CVD [1-8]. Lowering of LDL cholesterol levels by statin treatment has been the main therapeutic strategy for years. Statins resulted in improved mortality due to atherosclerosis, however, CVD events were only reduced by around 30%, which leaves an urgent need for new therapeutic strategies [9-11]. One possible way is to target the HDL-driven reverse cholesterol transport (RCT) pathway. The RCT pathway is a potential attractive target for reducing atherosclerosis because it consists of different organs and numerous transporters and receptors that can be modulated. It has also additional value to LDL lowering strategies, since it can not only prevent the accumulation of pro-atherogenic apoB-containing lipoproteins in the vessel wall but also reverse this process and thereby reduce lesion burden, at least in animal models[12]. However, to develop new effective therapies we must first have a better understanding of the reverse cholesterol transport pathway to identify new therapeutic targets.

What is reverse cholesterol transport?

Classic RCT is a process that comprises all the different steps in cholesterol metabolism between cholesterol efflux from lipid-laden cells until the final step, excretion into the feces. It involves (i) HDL-mediated efflux of cholesterol from lipid-laden macrophages in the arterial wall, (ii) uptake of this HDL cholesterol into the liver, and (iii) excretion of this HDL cholesterol into the bile and ultimately into the feces. The cholesterol can be excreted into feces as neutral sterols or (after metabolic conversion) as bile acids. It might be possible to positively stimulate overall RCT by modulating one or more steps within the RCT pathway. Therefore, a better understanding of the pathway may lead to new therapeutic targets in the prevention or cure of atherosclerosis. Chapter 2-5 of this thesis describe research carried out on different parts of the reverse cholesterol transport pathway in our quest to provide such improved insights.

How can we measure RCT?

In 2003 Rader and colleagues introduced a novel in vivo method to trace the movement of cholesterol all the way from the macrophage foam cells to plasma, liver, and ultimately, feces [13]. In short, J774 macrophages were loaded in vitro by incubation with acetylated LDL, labeled with 3H-cholesterol, and then injected intraperitoneally into recipient mice. Plasma samples were taken at several timepoints and feces were collected continuously for the duration of the experiment. The amount of 3H-tracer that is recovered in the feces is defined as completed RCT [13, 14].
The technique to measure macrophage-to-feces RCT has become a frequently applied method for which different types of macrophages can be used. Because of the shortcomings of using cell lines, all RCT experiments described in this thesis were carried out using primary macrophages obtained from the peritoneal cavity of donor mice.
There is currently no generally accepted method to asses RCT in humans. However, Rader and colleagues developed a macrophage-specific method using 3H-cholesterol/albumin complexes and tested this method in thirty subjects. The preliminary data that supports the feasibility of this approach in humans were presented so far only at conferences [55] and in the form of a patent but have not been formally published as a full scientific article.

Biliary cholesterol secretion and RCT

There are three ways to eliminate sterols from the body, by (i) the liver via biliary sterol secretion either as cholesterol or bile acids, by (ii) the intestine by way of modulation of sterol absorption rates as well as by (iii) transintestinal cholesterol efflux. **Chapter 2** of this thesis focused on the regulation of biliary cholesterol secretion, since this is a critical step in RCT [15]. There are several transporters localized to the bile canaliculus of hepatocytes that play essential roles in the biliary secretion process. To efficiently secrete biliary sterols mixed micelles are needed. Mixed micelles are formed by phospholipids and bile acids. For the biliary output of phospholipids and bile acids adenosine triphosphate (ATP)-binding cassette transporter B4 (ABCB4) and ABCB11, respectively, are required [16]. How are mixed micelles formed? Amphipathic bile acids are actively secreted by ABCB11 into the canalicular lumen where they form simple micelles in the aqueous environment of the bile whereas phospholipids are translocated to the outer leaflet of the canalicular membrane by ABCB4 and subsequently added to the simple micelles resulting in mixed micelles [17, 18].
The obligate heterodimeric transport proteins ATP-binding cassette subfamily G member 5 and 8 (ABCG5/G8) contribute quantitatively the major amount of cholesterol secretion into the bile [19-21]. In ABCG5 and/or ABCG8 knockout mice biliary cholesterol secretion is reduced by ≈75% [22-24]. This means about 25% of

biliary cholesterol secretion is independent of ABCG5/G8. What transporter might contribute to the ABCG5/G8 independent part of biliary cholesterol secretion? Scavenger receptor class B type I (SR-BI) mediates the uptake of HDL cholesterol into the hepatocyte, however, it is not only present on the basolateral membrane but also on the apical membrane of the hepatocyte [24]. Upon overexpression of SR-BI biliary cholesterol secretion is substantially increased independent of ABCG5/G8 [24, 25]. Also, Sr-bI knockout mice show a decrease in biliary cholesterol secretion rates and this translated into decreased RCT [25-27]. **Chapter 2** of this thesis describes our study that tested the hypothesis that SR-BI is largely responsible for the ABCG5/G8-independent part of biliary cholesterol secretion. To test this hypothesis Abcg5/Sr-bI double knockout mice were generated and characterized in comparison to wild-type controls and the respective Sr-bI and Abcg5 single knockout mice. Under steady-state conditions biliary cholesterol secretion is indeed significantly decreased in Sr-bI/Abcg5 double knockout mice in comparison to Abcg5 single knockout mice. This indicates that Sr-bI contributes a substantial part to the Abcg5/g8-independent biliary cholesterol secretion.

Since biliary cholesterol secretion is lower in Sr-bI/Abcg5 double knockout mice under steady-state conditions the effect of maximally stimulating biliary cholesterol secretion by bile acid infusion was also determined. Interestingly, the differences under steady-state conditions are no longer apparent under bile acid-stimulated conditions. Sr-bI knockout mice even display a trend towards increased biliary cholesterol secretion under bile acid stimulated conditions and the mechanism underlying this observation is currently unclear. In the Sr-bI/Abcg5 double knockout mice Abcg5 is clearly rate-limiting. The absence of Abcg5 prevents any significant increase in biliary cholesterol secretion rates in response to bile acid infusion.

Since biliary cholesterol secretion was decreased in Sr-bI/Abcg5 double knockout mice in comparison to Abcg5 single knockouts a macrophage-to-feces RCT experiment was performed comparing Abcg5 single knockout with Sr-bI/Abcg5 double knockout mice. This experiment showed the impact of Sr-bI on RCT independent of Abcg5. Importantly, there was a significant reduction of total macrophage-to-feces RCT by almost 50% in Sr-bI/Abcg5 double knockout mice. The data in **chapter 2** thereby demonstrate that the additional independent impact of SR-BI deficiency on biliary cholesterol secretion in mice that lack Abcg5 expression translates into decreased RCT. On the other hand, complementing these findings, the results in **chapter 5** indicate that hepatic Abcg5/g8 expression is less relevant for RCT, since modulating ABCG/5/G8 expression over a wide range resulted in corresponding substantial changes in mass biliary cholesterol excretion, but had no discernable effect on overall RCT.

In conclusion, data provided in chapters 2 and 5 demonstrate that under steady-state conditions SR-BI contributes significantly to the Abcg5/g8-independent part of biliary cholesterol secretion and deficiency in Sr-bI translates into a substantial decrease in RCT in Abcg5 knockout mice. However, under bile acid-stimulated conditions Abcg5/g8 were clearly rate-limiting for biliary cholesterol secretion indicat-

ing the importance of this transporter for mass cholesterol secretion into the bile. Combined, these data add to the current concepts on the differential roles and functionalities of SR-BI and ABCG5/G8 in the biliary sterol secretion process. Especially hepatic SR-BI seems to be a critical player with respect to RCT and further studies are warrented to explore how modulating SR-BI expression could be exploited as a therapeutic target for CVD.

The role of apolipoprotein E (apoE) in lipoprotein metabolism and atherosclerosis

ApoE is a protein that is predominantly produced and secreted by the liver [28]. However, it is also expressed in various peripheral tissues including macrophages [29, 30]. Several different studies indicate that ApoE plays a major role in lipoprotein metabolism and atherosclerosis. On the one hand, loss of function of apoE in mice as well as in humans is associated with increased atherogenesis [31, 32]. Conversely, overexpression of apoE in different mouse models has been shown to protect against the formation of atherosclerotic lesions [33-38]. Next to effects on the clearance of remnants, apoE has been reported to interact with ABCA1 and thereby promote cholesterol efflux from macrophages towards HDL [39]. In addition, it was also suggested that a lack of macrophage apoE might decrease overall RCT [40]. Because of these properties the effect of hepatic overexpression of human apoE on liver lipid metabolism, biliary sterol secretion and in vivo macrophage-to-feces RCT was investigated. **Chapter 3** of this thesis describes the data that were obtained from hepatic human apoE3 overexpressing mice. The obtained results demonstrate that hepatic overexpression of human apoE promotes SR-BI-mediated selective uptake of HDL cholesterol into the liver. However, this increase in selective uptake into the liver increased hepatic cholesterol content but did not translate into changes in biliary cholesterol secretion or fecal sterol excretion. Consequently, hepatic apoE overexpression also did not affect macrophage-to-feces RCT. It has been suggested that inhibition of hepatic ABCA1 activity by probucol reduces ABCA1-mediated cholesterol efflux from hepatocytes, thereby increasing cholesterol secretion into the bile and consequently the feces [41]. We therefore hypothesized that potentially increased ABCA1-mediated cholesterol efflux from hepatocytes might explain the unchanged biliary output, fecal output and RCT in apoE overexpressing mice. As described in **chapter 3**, probucol treatment indeed increased biliary cholesterol secretion as well as fecal cholesterol excretion in apoE overexpressing mice. Because probucol enhanced biliary and fecal sterol excretion it was investigated whether this would translate into an improvement of overall macrophage-to-feces RCT. Consistent with the mass output data, probucol significantly increased total macrophage-to-feces RCT. In summary, in **chapter 3** it is demonstrated that hepatic overexpression of human apoE not only facilitates SR-BI-mediated selective uptake of HDL cholesterol into the liver but also increases ABCA1-mediated resecretion of RCT-relevant cholesterol

back into the plasma compartment. Therefore, inhibiting hepatic ABCA1 might be a therapeutic strategy to enhance the anti-atherosclerotic property of apoE although the underlying mechanism needs to be delineated in more detail. Since hepatocyte ABCA1 is a major source for the generation of plasma HDL (75%), it would also in a more general context be interesting to explore what the diferential effects of decreasing liver ABCA1 are on mass HDL-C levels versus functional importance of the RCT pathway.

The emerging role of the intestine in RCT

As mentioned previously, cholesterol within apoB-containing lipoproteins is a major risk factor for the development of CVD. Circulating apoB-containing lipoproteins within the plasma compartment are determined by the combination of hepatic very low density lipoproteins (VLDL) production and intestinal production of chylomicrons. The microsomal triglyceride transfer protein (MTP) is essential for the assembly of both forms of apoB-containing lipoproteins [42-44]. This key role of MTP in determining circulating levels of apoB-containing lipoproteins and their importance for atherosclerosis resulted in the development of systemically active pharmacological inhibitors [45, 46]. Also, antisense oligonucleotides (ASOs) have been developed that specifically target hepatic MTP expression to lower pro-atherogenic apoB-containing lipoproteins in the plasma compartment [47]. The metabolism of triglyceride-rich lipoproteins such as VLDL is linked to HDL metabolism on several levels. First, lipolysis of VLDL by LPL results in the formation of pre-ß HDL, poorly lipidated apoA-I and thus increases plasma HDL-C levels [48]. Second, an intrahepatic link between VLDL and HDL was identified, namely that SR-BI-mediated hepatic uptake of HDL-C increases VLDL production and, importantly, at least partly mediates the resecretion of HDL-C within nascent VLDL particles [49]. However, it is not known how pharmacological MTP inhibition or decreased hepatic MTP expression affect the rerouting of cholesterol between different compartments and pathways. In **chapter 4** it is described how decreasing systemic and hepatocyte-specific MTP activity impact cholesterol metabolism and RCT. It was demonstrated that decreasing both intestinal and hepatic secretion of apoB-containing lipoproteins by pharmacological inhibition of MTP increases RCT. In contrast to systemic MTP inhibition genetic hepatocyte-specific MTP ablation decreases RCT. These data are consistent with a concept that hepatic secretion of RCT-relevant cholesterol within apoB-containing lipoproteins contributes to RCT, however, upon systemic MTP inhibition by pharmacological means the effect on RCT of the decrease in intestinal cholesterol absorption is relatively larger than the decrease in VLDL production. How can these results be explained? The most likely explanation is the decrease in VLDL production itself, since HDL-derived cholesterol can be resecreted within VLDL [49]. Although biliary secretion plays the predominant role in RCT it was shown in Abcb4 knockout mice which virtually lack biliary cholesterol secretion that about 25% of the macrophage-derived counts were still excreted [15]. The transin-

testinal cholesterol excretion (TICE) pathway is increasingly gaining interest. HDL have been excluded by several studies as lipoprotein particles donating cholesterol for TICE [15, 50]. Therefore, apoB-containing lipoproteins, including VLDL, likely serve as the main cholesterol donor particle for this pathway [50, 51]. The decrease in RCT in liver-specific MTP-deficient mice could thus be explained as follows: (i) a fraction of RCT-relevant cholesterol enters the liver via HDL and is (ii) resecreted within VLDL, which is then (iii) subsequently taken up by the intestine for final excretion into the feces via TICE. On the other hand, inhibiting intestinal MTP resulted in decreased cholesterol absorption **(chapter 4)**. Experiments with the cholesterol absorption inhibitor ezetimibe indicated that this is also an important factor to increase RCT [52, 53].
The combined experiments described in **chapter 4** indicate that the intestine contributes to RCT in two different ways, by reabsorption of RCT-relevant cholesterol secreted via the bile as well as likely by excreting RCT-relevant cholesterol that is secreted by hepatocytes within VLDL via the TICE pathway.

The impact of ABCG5/G8 on RCT

As mentioned above, the obligate heterodimer ABCG5/G8 is responsible for approximately 75% of mass biliary cholesterol secretion. However, it is not only expressed in the liver but also in the small intestine where it resecretes absorbed plant sterols and cholesterol back into the intestinal lumen. In **chapter 5** we show that modulating the hepatic expression of ABCG5/G8 over a wide range, from knockout to several fold overexpression translates into corresponding changes in biliary cholesterol secretion from a 75% decrease to a 500% increase compared with wild-type mice. However, despite these substantial differences, overall RCT remained unaltered. Thereby the results in **chapter 5** indicate that hepatic Abcg5/g8 expression is of minor importance for RCT and that in the case of ABCG5/G8 mass biliary cholesterol secretion is dissociated from RCT. But how can this be explained? It is conceivable that RCT-relevant cholesterol is secreted from a hepatic cholesterol pool that is less accessible to ABCG5/G8, but more to SR-BI **(chapter 2)**. Taken together the data summarized in **chapter 5** indicate that modulating hepatic ABCG5/G8 expression is likely not a successful approach to increase RCT and decrease atherosclerotic CVD. However, more research is still necessary to study the intracellular nature of these differential, up to now hypothetic, intrahepatic cholesterol pools and the modes of their regulation.

Conclusion

How can we integrate the combined data from **chapters 2-5** into a single model? By combining classic RCT with the TICE pathway. HDL particles elicit cholesterol efflux from macrophage foam cells and transport this cholesterol back to the liver. HDL-

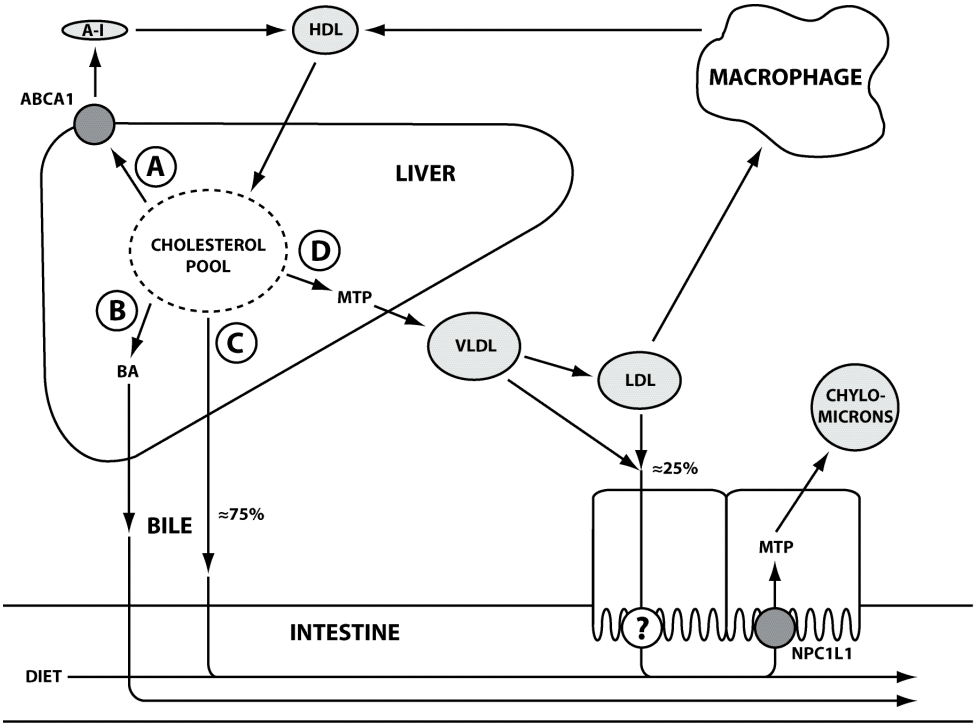

Figure 1: Schematic integration of the results of current concepts of cholesterol metabolism. HDL particles elicit cholesterol efflux from macrophage foam cells and transport this cholesterol to the liver where it is taken up (reverse cholesterol transport pathway). HDL-derived cholesterol within the hepatocyte can then either (A) be resecreted via ABCA1 to lipidate apoA-I resulting in the de novo formation of HDL, or (B) be converted into bile acids, or (C) be directly secreted into bile or (D) be resecreted as part of VLDL in a MTP-dependent process. VLDL or LDL, which is currently unclear, likely represent the main substrate for transintestinal cholesterol excretion (TICE). For RCT-relevant cholesterol entering the intestinal lumen the biliary route is apparently a major contributor, our estimation is around 75%, while 25% is derived from TICE. Intestinal MTP is important for cholesterol absorption resulting in increased fecal cholesterol excretion upon MTP inhibition. Please note that it has not been formally excluded that also HDL might serve as a substrate for TICE.

derived cholesterol within the hepatocyte can be rerouted in different ways. It can be resecreted into the plasma compartment via ABCA1 to lipidate apoA-I resulting in de novo formation of HDL (Figure 1A), or it can be converted into bile acids before secretion into the bile (Figure 1B), or it can be directly secreted into the bile (Figure 1C), or it can be resecreted as part of VLDL in a MTP-dependent process and thereby likely represent a substrate for TICE (Figure 1D). We estimate from the current work and previous studies that around 75% of RCT-relevant cholesterol not converted into bile acids is entering the intestinal lumen via the biliary route, while 25% is derived from TICE. To therapeutically exploit this knowledge for clinical use further studies are required.

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Appendices

Samenvatting

Wereldwijd zijn hart- en vaatziekten (oftewel cardiovasculaire aandoeningen) de belangrijkste doodsoorzaak. Onderzoek heeft aangetoond dat cardiovasculaire aandoeningen en de concentratie low density liporotein (LDL) cholesterol in het bloed direct gecorreleerd zijn. Daarnaast is aangetoond dat het risico op cardiovasculaire aandoeningen omgekeerd evenredig is met de concentratie high density lipoprotein (HDL) cholesterol in het bloed. Atherosclerose (aderverkalking) is een aandoening waarbij de bloedvaten dichtslibben. Dit begint met het binnendringen van LDL-cholesterol in de vaatwand. Vervolgens zullen macrofagen proberen om het cholesterol op te ruimen. Echter, als er te veel cholesterol aanwezig is, zullen de macrofagen verzadigd raken. De verzameling verzadigde macrofagen vormt dan een plaque (oftewel een afzetting). Wanneer deze plaque blijft groeien, kunnen blokkades in de bloedvaten ontstaan, met alle gevolgen van dien.

Reverse cholesterol transport (RCT) is een proces bestaande uit alle stappen in cholesterol metabolisme van cholesterol efflux uit de macrofagen tot aan de excretie in de feces (macrofaag-naar-feces RCT). De eerste stap van de RCT-route is de efflux van cholesterol uit de macrofagen in de vaatwand (plaques) naar HDL-deeltjes. Vervolgens wordt het HDL-cholesterol terug getransporteerd naar de lever en door de lever opgenomen. De derde stap is de uitscheiding van dit cholesterol in de gal, om uiteindelijk in de feces te belanden. Het is goed mogelijk dat één of meerdere stappen van de RCT-route te beïnvloeden zijn, waardoor het proces verbeterd kan worden om zo de plaquevorming tegen te gaan. Een beter begrip van de RCT-route is dus van essentieel belang voor de preventie of genezing van atherosclerose. In hoofdstuk 2 tot en met 5 van dit proefschrift zijn verschillende onderdelen van de RCT-route onderzocht.
Scavenger receptor class B type I (SR-BI) is een transporteiwit in de lever dat de selectieve opname van HDL-cholesterol medieert. ATP-binding cassette subfamily G member 5 en 8 (ABCG5/G8) is een transporteiwit in de lever dat verantwoordelijk is voor ongeveer 75% van de totale cholesterol secretie in de gal. Dit betekent dat ongeveer 25% van de cholesterol secretie in de gal onafhankelijk van ABCG5/G8 plaatsvindt. In hoofdstuk 2 is de hypothese getest dat SR-BI grotendeels verantwoordelijk is voor de ABCG5/G8 onafhankelijke cholesterol secretie in de gal. Om dit te testen is een muismodel gebruikt dat de transporteiwitten SR-BI en ABCG5/G8 niet heeft. Uit de resultaten blijkt dat SR-BI bijdraagt aan de ABCG5/G8 onafhankelijke cholesterol secretie in de gal en ook dat SR-BI bijdraagt aan macrofaag-naar-feces RCT.
Apolipoproteïne E (apoE) is een eiwit dat hoofdzakelijk door de lever wordt geproduceerd en een belangrijke rol speelt bij atherosclerose. In hoofdstuk 3 is bestudeerd wat het effect is van overexpressie van apoE in de lever van muizen op cholesterol secretie in de gal en macrofaag-naar-feces RCT. Uit de resultaten blijkt dat de door SR-BI gemedieerde selectieve opname van HDL-cholesterol in de lever toeneemt. Dit heeft echter geen invloed op macrofaag-naar-feces RCT. De verklaring hiervoor

is dat apoE zorgt voor een verbeterde werking van ATP-binding cassette transporter A1 (ABCA1). ABCA1 is een transporter die het van HDL afkomstige cholesterol teruggepompt van de lever naar het bloed om nieuw HDL te vormen met apolipoproteïne A-I (apoA-I).
Cholesterol in apolipoproteïne B (apoB) bevattende lipoproteïnen, zoals LDL, zijn een risicofactor voor hart- en vaatziekten. ApoB bevattende lipoproteïnen worden op twee plaatsen geproduceerd. Ten eerste in de lever in de vorm van very low density lipoprotein (VLDL) cholesterol en ten tweede in de dunne darm in de vorm van chylomicronen waar het cholesterol uit het voedsel wordt ingebouwd. Voor zowel de productie van VLDL als chylomicronen is microsomal triglyceride transfer protein (MTP) nodig. In hoofdstuk 4 is bepaald of het systemisch remmen van MTP (dus zowel in de lever als in de dunne darm) of het remmen van MTP alleen in de lever van muizen invloed heeft op RCT. Uit de resultaten blijkt dat RCT wordt verhoogd door het systemisch remmen van MTP en dat het alleen in de lever remmen van MTP RCT verlaagt. Deze resultaten doen vermoeden dat VLDL een bijdrage levert aan RCT.
Zoals eerder genoemd, is ABCG5/G8 verantwoordelijk voor ongeveer 75% van de cholesterol uitscheiding in de gal. Bij muizen die geen ABCG5/G8 hebben, is de cholesterol secretie in de gal 75% lager. Bij muizen waarbij ABCG5/G8 tot overexpressie wordt gebracht in de lever is de cholesterol secretie in de gal 500% hoger. In hoofdstuk 5 wordt aangetoond dat, ondanks deze verlaging of verhoging van cholesterol secretie in de gal, geen verandering in RCT optreedt.
Naast de RCT-route is er de trans intestinale cholesterol excretie (TICE) route waarbij cholesterol direct vanuit het bloed in de darm wordt uitgescheiden (dus niet via gal) om uiteindelijk in de feces te belanden. Tot nu toe werden deze routes als op zichzelf staande routes beschouwd. Maar door de resultaten van hoofdstuk 2 tot en met 5 te integreren in één enkel model is de RCT-route mogelijk te combineren met de TICE-route. HDL-deeltjes in het bloed mediëren de efflux van cholesterol uit de macrofagen in de plaques. Vervolgens wordt dit HDL-cholesterol naar de lever vervoerd, waar het via SR-BI in de lever wordt opgenomen. Vanuit de lever kan dit cholesterol (i) via ABCA1 worden uitgescheiden in het bloed waar het met apoA-I nieuw HDL vormt, (ii) worden omgezet in galzouten voordat het in de gal wordt uitgescheiden, (iii) direct in de gal worden uitgescheiden of (iv) met behulp van MTP worden uitgescheiden in het bloed als onderdeel van VLDL waarna het vermoedelijk in de TICE-route terechtkomt.

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Biografie

Arne Dikkers werd op 13 februari 1979 geboren in Groningen en groeide op in Nuis, een dorp in het westen van de provincie Groningen. Hij behaalde in 1999 zijn vwo-diploma aan het Nienoord College in Leek. Vervolgens startte hij met de studie (Medische) Biologie aan de Rijksuniversiteit Groningen, die hij afrondde in 2008. Tijdens zijn studie heeft hij bovendien de lerarenopleiding afgerond. In 2009 startte hij met zijn promotieonderzoek op de afdeling Kindergeneeskunde van het Universitair Medisch Centrum Groningen, onder begeleiding van Prof. dr. Uwe J.F. Tietge. Zijn promotieonderzoek werd gefinancierd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO). De resultaten van het onderzoek staan beschreven in dit proefschrift.

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